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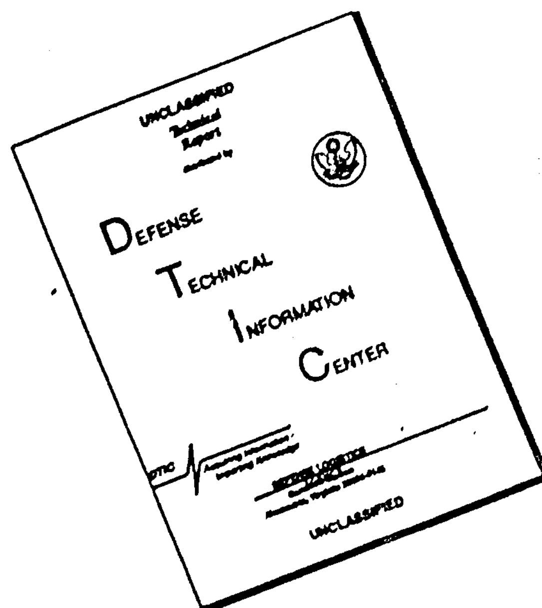

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STUDIES ON TICK-BORNE ENCEPHALITIS AND OTHER ARTHROPOD-BORNE VIRUS DISEASES

Final Technical Report

By

Ch.Kunz,M.D., H.Aspöck,Ph.D., W.Frisch-Niggemeyer,Ph.D.,
H.Hofmann,M.D., A.Radda,Ph.D., G.Wiedermann,M.D.

July 1971

EUROPEAN RESEARCH OFFICE
Contract Number DAJA37-70-C-2462

Hygiene-Institut der Universität
Kinderspitalgasse 15
1095 Wien, Austria

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Abstract

Three new foci of TBE virus were found in Lower Austria. In studies aimed at interrupting the virus cycle in nature Gardona^{R} was very effective against ticks. Effect of the small mammal control program in Carinthia is not yet clear.

Attachment of TBE virus to its receptor (TPI) is a two-step reaction consisting first of a loose electrostatic and later a stronger binding. TPI inhibits the infectivity for mice of group B but not of group A arboviruses. Antibodies against TPI were obtained in rabbits.

In animal experiments no conclusive evidence was obtained that formation of RNAse is one of the defense mechanisms against arboviruses.

Interferon does not account for the resistance of house mice to fatal TBE.

Tilorone $^{(R)}$HCl protects white mice against TBE. Combined application of this compound with Poly I:C did not improve the results.

320 cases of TBE were diagnosed in Austria. The disease can rapidly be diagnosed by demonstration of IgM-antibodies in the patient's serum with 2-Mercaptoethanol.

Interferon can be demonstrated in the CNS of patients who succumb to TBE provided death occurs soon after onset of disease.

Tahyna and Calovo viruses are regularly active on the Eastern side of Neusiedlersee but not on the Western side of the lake. No evidence was found for the hibernation of Tahyna virus in Culex modestus.

For the first time antibodies against Tahyna virus were found in birds (Sturnus vulgaris). hares (Lepus europaeus) are not susceptible to Calovo virus.

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Tick-borne encephalitis (TBE)

Field studies

(1) Introduction

In 1969 and 1970, field studies were continued in order to locate new foci of TBE virus. This was done by virus isolation experiments from ticks collected in woods that had been visited by persons prior to their becoming ill with TBE. Thus several foci in Upper Austria and Carinthia were found. In 1970 and 1971 again questionnaires were sent to patients with TBE. According to the information obteined we continued to search for new foci of TBE virus in Lower Austria and in Burgenland. Also surveillance of TBE in the three known foci in Lower Austria, Gfieder, Strelzhof/Willendorf end Hernstein was continued.

Our present field studies are done with the aim of finding methods that could lead to the interruption of the virus cycle in nature. This goal could be achieved by measures directed either against the tick vector or the vertebrate reservoir of the virus.

One line of investigations deals with the question of whether or not a sufficient reduction of ticks can be achieved with modern insecticides. For this purpose Shell's Gardona(R) which is an organophosphorus compound was tested for its effectiveness against ticks in three different locations.

The other main line of research developed out of ~~our~~ finding that small mammals are essential for the persistence of TBE virus in a focus. First experiments are being conducted in the Teggenbrunn focus in Carinthia with the hope that a reduction of the population density of these vertebrates will be detrimental to the development of nymphs and eventually stop cycling of the virus.

(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 1-5 individuals respectively. They were suspended in a medium consisting of PBS and 10% horse serum

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Tick-borne encephalitis (TBE)

Field studies

(1) Introduction

In 1969 and 1970, field studies were continued in order to locate new foci of TBE virus. This was done by virus isolation experiments from ticks collected in woods that had been visited by persons prior to their becoming ill with TBE. Thus several foci in Upper Austria and Carinthia were found. In 1970 and 1971 again questionnaires were sent to patients with TBE. According to the information obtained we continued to search for new foci of TBE virus in Lower Austria and in Burgenland. Also surveillance of TBE in the three known foci in Lower Austria, Gfieder, Strelzhof/Willendorf and Hernstein was continued.

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The other main line of research developed out of our finding that small mammals are essential for the persistence of TBE virus in a focus. First experiments are being conducted in the Taggenbrunn focus in Carinthia with the hope that a reduction of the population density of these vertebrates will be detrimental to the development of nymphs and eventually stop cycling of the virus.

(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 1-5 individuals respectively. They were suspended in a medium consisting of PBS and 10% horse serum

and inoculated intracerebrally into baby mice. The animals were observed for 12 days.

Gardona(R): Tests were done in order to develop a control program against ticks with Gardona(R). During the fall excursion on September 25 and 26 in 1970, the 16 control points in our study area near Hernstein were searched for ticks in the usual manner. Then around seven of these check points, each covering an area of 16 m^2, the ground and underwood was sprayed with a solution of Gardona(R) at a concentration of 1 lb. per acre. On the following day and 12 days thereafter ticks were again collected around the 16 poles.

In the spring of 1971 in an afforestation near the area mentioned above 8 fields of 25 m^2 each were marked. Four of these fields were sprayed with Gardona(R) as described above. Tick collections were made before spraying and on the 3rd and 8th days thereafter. A similar experiment was done in a wood near the Danube river at Mühlleiten, a few miles east of Vienna.

Small mammals: Eradication of the virus by trapping of small mammals is being attempted in the focus of Taggenbrunn (Carinthia). This focus was chosen to test our hypothesis that small mammals are essential for the persistence of TBE in a focus. So far, four excursions were made in order to trap small mammals. About 120 small mammal traps were set up for 1 to 3 nights.

(3) Results

Gfieder: In 1970 from June 17-20, 141 nymphs and 25 adults of Ixodes ricinus were collected. In the fall excursion (September 20-21) 149 nymphs and 14 adults (Ixodes ricinus) were collected. From these ticks no virus was isolated (Table 1a).

Strelzhof/Willendorf: During the spring excursion (June 20-21) a total of 289 nymphs and 60 adults of Ixodes ricinus as well as one specimen of the tick species Haemaphysalis concinna could be collected. Out of one pool consisting of 23 nymphs and another pool consisting of 3 males (all ticks stem from control point 11) two strains of TBE virus could be isolated. The fall excursion was done on September 29-30. Out of 479 nymphs and 17 adults of Ixodes ricinus no virus could be isolated (Table 1a).

Hernstein: In the spring excursion made on June 6-7, altogether 1014 nymphs and 104 adults of Ixodes ricinus as well as one nymph of Haemaphysalis concinna were collected.

No virus isolation was possible. Also virus isolation from 235 nymphs and 13 adults (Ixodes ricinus), collected during the fall excursion (September 24-25), was not successful (Table 1a).

New foci in Lower Austria: According to the information obtained from patients with TBE ticks were collected in different woods in Lower Austria which are listed in Table 2. It will be seen that of the 9 different locations which we visited in the spring of 1971, Hohenegg II, Neudörfl and Völtendorf could be confirmed as foci of TBE virus. In Hohenegg II the virus strain was isolated from a pool of 3 males and 1 female of Ixodes ricinus. This focus is of the hercynic type and is situated near the ruin of Hohenegg. It is of special interest that in 1970 three persons and in 1971 two persons obviously acquired infection in Hohenegg, indicating that this is a permanent focus. The focus of Neudörfl was investigated because this year three persons who came down with TBE had visited this area within the period of incubation. Also in this focus which belongs to the hercynic type virus isolation was made from a pool of 3 males and 2 females of Ixodes ricinus. In the Völtendorf forest where the third focus was located one person contracted infection in May 1971. We isolated 2 strains of TBE virus from 20 and 23 nymphs respectively (Table 1 b).

Gardona(R): The results of the field study with Gardona(R) are listed in Tables 3 and 4 and in 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 Gardona(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 control field. In Hernstein I ticks were reduced by approximately 80% 12 days after spraying and in Hernstein II, where conditions permitted a more even distribution of Gardona(R) results were still better. The most drastic effect was achieved in Mühlleiten where undergrowth is dense and mainly consists of herbs and shrubs and not of young trees and bushes as in the other two areas.

Small mammals: The results of trapping of small mammals are presented in Table 5. It can be seen that the population density was rather low already in the spring of 1970. This was probably caused by the long and cold winter of 1969/70. Further investigations will show if keeping the number of small mammals low will lead to the eradication of the focus as intended by us.

In 1970 there was still plenty of virus in the focus as it was indicated by the fact that 6 sheep, which were pastured in the focus during summer, all had antibodies in the neutralization test when they were bled in December.

(4) Discussion and Conclusions

Surveillance of TBE virus in 3 known foci in Lower Austria again showed that permanent foci usually consist of microfoci in which the virus comes and goes. As it is evident from the Strelzhof focus where virus was isolated from ticks collected around check point 11, frequently the virus reappears in exactly the same spot where it had been found previously (Radda et al.1968), (1).

Recently we have started to search for new foci on the basis of informations obtained from patients. This is done with the purpose of finding foci that are suitable for control programs directed either against ticks or mammals. One of the 3 new foci found appears to meet the requirements for a successful application of Gardona(R).

The results of our first tests done with this insectizide are very encouraging, and we are hopeful that our future studies will prove its effectiveness also in permanent foci. In the near future a field trial will be done in the focus of Hochosterwitz (Carinthia) where especially high rates of virus-carrying ticks are found as stated in the Last Final Technical Report (2).

Control of small mammals as reservoir animals of the virus will also be continued. In connection with this it would be desirable to find methods which are more effective then trapping.

(5) Summary

Surveillance of TBE virus in known foci confirmed earlier observations that within these areas microfoci exist in which the virus comes and goes. Three new foci were located in Lower Austria by virus isolation from ticks.

Gardona(R) is effective against Ixodes ricinus ticks. In 3 field studies a reduction of ticks ranging between 80% and almost 100% was achieved. The control program of small mammals in Taggenbrunn (Carinthia) was continued. Population density of these reservoir animals of TBE virus was kept low. It remains to be seen if this measure is effective against the cycling of the virus.

Experimental laboratory Investigations

(1) Studies on the receptor substance (RS) for TBE virus and other arboviruses

(1.1) Investigations on the dynamics of the reaction between TBE virus and its specific receptor substance (RS)

Introduction and earlier results:

We developed a method for testing the competitive inhibition of the hemagglutination (HA) of arboviruses (3). Using this procedure for monitoring, it was possible to measure the activity of arbovirus receptor preparations, extracted from frozen brain by lipid solvents (4). These lipid extracts could be fractionated (4,5), the active component was purified and its chemical structure was identified (2,6). The substance, a triphosphoinositide (TPI), was a very strong inhibitor of the HA and also of the infectivity of group B arboviruses (Flavi-viruses). Apparently, it did not react with arboviruses of group A (Alphaviruses). We regard it to be the receptor substance (RS) for arboviruses of group B (2,7).

Preparation of receptor substance:

The preparation of 90% pure triphosphoinositide (TPI) from frozen brain is now a routine procedure in our laboratory. It is performed as described in the last Technical Report (2). By doing the final steps, i.e. precipitation with acetone and chromatography over Sephadex, in rather concentrated solution and by adding a trace of $CaCl_2$ during this precipitation, the final yield could be slightly increased. From about 45 monkey brains we were able to prepare nearly 1000 mg of purified RS (Table 6).

Reaction of TBE virus with purified RS:

A series of experiments was undertaken to study the conditions of the competitive inhibition of TBE virus hemagglutination by purified TPI.

Four HA-units (HAU) of TBE virus were incubated with a dilution series of TPI for 15 min. at $0^{\circ}C$ and at different pH values. Then erythrocytes were added in a buffer, which, after addition to the reaction mixture, yielded a pH of 6.4, the optimal pH value for hemagglutination. From

Table 7 it can be seen that there is a strong inhibition of the HA at low pH values up to a pH of 7.4. Further increase in pH value required larger amounts of TPI for total inhibition of the HA, but even at pH 9 it was possible to interfere with the HA when comparatively high concentrations of TPI were applied.

The influence of temperature was also investigated. At pH 7.4 where no decreases of the HA titer of TBE virus due to acidity alone occurs (8) tests at different temperatures could be performed. Four HA units of TBE virus were incubated with a dilution series of TPI at 0°C, 22°C and 37°C for 15 min. and afterwards erythrocytes were added in the same manner as described for the previous experiment. From Table 8 it is obvious that the inhibition of HA works best at low temperatures.

A similar experiment was performed with the modification that the TPI dilutions were allowed to react with TBE virus at a constant temperature of 0°C but at varying periods of time. Table 4 shows that even rather short exposure of the virus to TPI-Ca resulted in inhibition of the HA. This points to a rapid reaction between the virus and its RS. Contrary to our expectations, the inhibition was slightly less at longer incubation time.

The decrease of receptor activity at longer intervals and also at higher temperatures could perhaps be due to a "receptor destroying enzyme (RDE)" on the surface of the arbovirus particle, analogous to the neuraminidase of myxoviruses. However, the presence of an RDE - which in this case would be a phospholipase - does not necessarily mean that it is an integral part of the virion. It could just as well be of host origin and only be adsorbed onto the virus particle or even be present independently in solution. Finally, it could also be that a nonenzymatical hydrolysis of the RS occurs under the described condition.

The complex of TBE virus with its RS is separable by the addition of certain strongly basic molecules as streptomycin or protamin. To 0.5 ml virus suspension at pH 7.4, containing about 2000 HAU, equal volumes of RS were given at the same pH. The amounts of RS were 12 µg, 25 µg and 50 µg. A control was performed with buffer containing no RS. The virus was allowed to react with the receptor at 0°C for 3 min. After this period, the reaction is complete (Table 9). Then 1 ml of a solution of either streptomycin sulfate or protamin sulfate (salmin) in the same buffer of pH 7.4 was added to each tube. The streptomycin sulfate solution was made to 1.4 % which resulted in a final concentration of 0.01 M. Protamin sulfate was applied in a concentration of 1 mg/ml. After 10 min. at 0°C the mixture was centrifuged and the HA titer of the supernatant was measured. From

Table 10 it can be seen that neither streptomycin nor protamin alone did diminish the HA titer of the virus preparation. A suitable amount of RS nearly totally suppressed the HA, but this inhibition could be largely neutralized by the addition of streptomycin or protamin. It must be concluded that the linkage between TBE virus and RS can be split by these basic compounds.

All the experiments described above were performed with a non-infectious but well hemagglutinating virus, prepared at 37°C (9). Using such an inactivated preparation, the virus bound to RS could be released by streptomycin when the complex had been formed by incubation at 0°C and pH 7.4 for longer periods. However, after reaction at higher temperature (37°C), there was an indication that the complex was not separable as easily as at 0°C (Table 11). Using fully infective virus instead of heat inactivated preparations, the stronger binding occurs rather rapidly even at 0°C. After 20 sec., more than 90% of the virus particles are strongly bound to the RS, i.e. they are not separable by the addition of streptomycin. After 1 min. about 95% are irreversibly combined. However, the last 1-2% need more than 10 min. to become strongly bound to the RS. It seems that this portion consists of inactivated virus particles.

Conclusions and Discussion:

The high speed of the reaction between TBE virus and its RS (TPI) and the ease of separation of the virus-receptor complex by strongly basic molecules leads to the conclusion that the first step of the interaction between TBE virus and TPI-Ca is of electrostatic nature. This attraction seems to be followed by a second step caused by stronger forces which are not any more susceptible to the interference of positively charged molecules.

An interaction between a cell-receptor and an arbovirus of group B which occurs in two steps has already been described by other authors (10). The receptor had been extracted from brain and from erythrocytes by aqueous solvents (11), but after extensive purification, it had a lipid content of 90% (12). Its activity was sensitive to phospholipase C and D. These authors were not able to identify this substance but concluding from our own results, we have no doubt that also in this case, the receptor active component was a triphosphoinositide.

Summary:

The dynamics of the reaction of TBE virus with purified receptor substance (RS) was studied. The RS, a triphosphoinositide, was extracted from brain yielding 30 mg/100 g on the average. Its reaction with TBE virus was studied using a

competitive HA-inhibition test. TBE virus reacted best with this substance at pH values between 6 and 7.4. At low temperatures and at short reaction periods smaller amounts of RS were able to inhibit the viral HA. This could be due either to chemical or to enzymatical degradation of the RS during the test. The complex of RS with heat inactivated TBE virus formed at 0°C could easily be separated by protamin and streptomycin. The splitting of a complex formed at 37°C was less easy and the complex of RS with infective virus became resistant to streptomycin in less than 20 sec. even at 0°C. It is concluded that TBE virus reacts with its RS in two steps. The first, caused by electrostatic forces, is followed by a stronger binding which is not separable by charged molecules. These conclusions are consistent with the observations of other authors using a different arbovirus B and a non-identified receptor.

(1.2) Inhibition of infectivity for mice of TBE virus by its receptor substance (RS).

In the last Final Technical Report (2) an inhibitory effect of the receptor substance TPI against West Nile virus was described. These studies were done with the plaque test in tissue cultures.

In a new series of experiments performed under the present contract it was investigated to which extent TPI is capable of neutralizing the infectivity of TBE virus for mice. In addition, Dengue virus type II which also belongs to the group B of arboviruses as well as the group A arboviruses Semliki and Sindbis were also studied.

In these experiments mouse brain suspensions of the viruses were purified and mixed with Ca-TPI as described in the Last Final Technical Report (2). After incubation at 0°C for 30 minutes these mixtures as well as control dilutions of the virus, adjusted to the same pH, were injected intracerebrally into baby mice (dose 0.02 ml).

It will be seen in Table 13 that a definite inhibition was achieved of TBE virus when 8 µg of Ca-TPI were allowed to react with amounts of virus ranging between 10^{1-8} LD_{50} and 10^{3-4} LD_{50}. This phenomenon was observed under pH conditions of pH 6.4-pH 7.5. At pH 7.5 the inhibitory effect was most pronounced leading to the neutralization of more than 99% of virus in the mixture. The virus did not react with the Ca-TPI when tested at pH 8.0 and 9.0.

Thus, as it is the case with hemagglutinin prepared from the virus also infectious viria can only adsorb to

Ca-TPI (=RS) within a certain range of pH which is, however, much wider with the latter preparation.

Table 13 also shows that when the amount of Ca-TPI was reduced to 1.6 µg the inhibitory effect went below 90%.

It is further evident from Table 13 that Dengue virus type II is also inhibited by Ca-TPI. This substantiates our results obtained with the HI test that this compound probably is the receptor substance of all group B viruses.

In our control experiment not listed in Table 13 it was learned that when lecithin was used instead of Ca-PPI TBE virus was not inhibited.

In Table 14 the results are summarized of tests conducted with Semliki and Sindbis viruses under different conditions of pH. From these tests it is evident that Ca-TPI does not neutralize the infectivity of these group A viruses. In no test a 90% inhibition which is the threshold of statistic significance was reached, rendering it unlikely that TPI acts as receptor substance for these viruses.

Thus the results of the infectivity-inhibition tests essentially parallel those of the inhibition tests described in previous reports. We can now state with great confidence that TPI is the receptor substance of probably all group B viruses.

Summary:

Ca-TPI inhibited the infectivity for mice of TBE and Dengue II viruses (arbo group B) but not of Semliki and Sindbis viruses (group A). This neutralizing effect was obtained within a wide range of pH. The results provide further prove that TPI is the receptor substance of group B arboviruses.

(1.3) Antibodies against the receptor substance.

Material and Methods:

Rabbits were immunized against TPI which is the receptor substance with the following mixture:

25 mg TPI
25 mg BSA (bovin serum albumin)
2.5 mg saline
2.5 ml complete Freund's adjuvant
0.5 cc of this mixture was injected subcutaneously in each of the pads. Blood was drawn after 17 days. Three days later a booster injection was given and the final blood sample was collected on the 28th day by heart puncture.

The complement-fixation (CF) test was performed as usual in plastic trays adding 1/4 of an antigen unit and $2'H_{100}$. The following preparations served as antigens:
a) Homogenized mouse brain (1 g/10 ml veronal buffer)
b) myelin of mouse brain according to Eichberg and Dawson (13).

This material was prepared by means of differential ultracontrifugation in

c) 1 γ TPI + 50 γ lecithin/ml
d) 27.5 γ TPI + 27.5 γ lecithin/ml
e) 27.5 γ TPI + 1 γ lecithin/ml
f) TPI acid in ethanol (96%) 0.11 mg/ml
g) sodium TPI on veronal buffer.

Cardiolipin flocculation test (VDRL) was done with a reagent from Behringwerks (Marburg).

The neutralization test was performed as usual with baby mice. The neutralizing effect of receptor substance was tested after incubation of virus suspension with TPI.

Anti TPI-sera were tested furthermore by means of a neutralization-inhibition test. For this purpose TPI (0.4 mg/ml) was incubated with test or control serum at room temperature for 1 hour and consecutively at 5°C for 15 min. with a virus-suspension (Hypre). This preparation was inoculated into baby mice as usual. All these preparations were adjusted to pH = 7.3.

Chromatography with Sephadex G-200 was performed according to Kielandor and Flodin (14).

Results and discussion:

Five rabbits were immunized with TPI and their serum subsequently was tested by means of the cardiolipin flocculation test. This was done by reason of the structural similarities of the lipids involved with TPI. Indeed all sera exhibited a positive flocculation test. The one (Nr.429) showing most pronounced reaction (2-3+) also revealed the highest titer in the CF against mouse brain (1:80), whereas the others only reacted with lower titers (1:40). Serum 429 was also tested against other antigens (see Table 15) and compared with a control and an anticerebroside serum.

The highest titers of the TPI sera were obtained with mouse brain suspension and myelin. With TPI-lecithin the titers were lower. The control serum exhibited no significant activity against any of the antigens tested. Anticerebroside sera reacted with mouse brain suspension and myelin only.

This finding speaks in favour of a specific reaction of anti-TPI serum. Absorption with TPI-Ca of the anti-TPI serum rendered the sample anticomplementary.

The activity of anti-TPI serum was also tested by means of the neutralization-inhibition test (see methods). We attempted to inhibit the virus-neutralizing effect of receptor substance (TPI) by addition of an anti-TPI serum (Tables 16 and 17). As can be seen from Table 16 the virus-neutralizing effect of TPI can be inactivated not only by addition of anti-TPI-serum, but also by anticerebroside and control serum from untreated rabbits. This effect was further investigated when fractions of these sera obtained by chromatography with Sephadex G-200 were used instead of whole sera (Table 17). This experiment shows that the nonspecific inhibitory activity of sera resides to a very high degree within the albumin fraction. A difference which seems to be significant could be observed between anti-TPI and control sera in that IgM fractions of test sera showed a stronger inhibition of the virus-neutralizing effect of TPI than IgM fractions of control sera (difference = 10^{-1}). This effect, although significant, is not an overwhelming one. It might well be however that sera with higher titer could be even more effective.

Several conclusions may be drawn from our experiments. First of all, antibodies against receptor substances are readily formed in rabbits. Apparently, they are specific, although titers are low and production of antisera with higher levels of antibodies is desirable. In the present situation blockade of cell receptors making them inaccessible to TBE virus does not seem a workable concept since the titer of antilipid antisera is a bit too low. Secondly, neutralization of virus by receptor-substance is possible, however only in vitro. In vivo TPI in its present stage of preparation will be readily adsorbed to albumin and inactivated. Thus TPI does not seem to be a useful therapeutic or prophylactic agent at the present time. Thirdly, the role of serum albumin in the inactivation of TPI is a very interesting point which should be further examined. We feel however that the binding of TPI by serum albumin is due to the affinity of serum albumin to lipids.

Summary:

Antibodies against the receptor substance (TPI) of TBE virus have been produced in rabbits. So far however, it has not been possible to obtain antisera with a titer high enough to achieve blockade of cellreceptors against the adsorption of TBE virus. The antisera are apparently specific as it was demonstrated in several in vitro tests. Neutralization of TBE virus by TPI in vitro is possible. This substance however does not seem to be a promising therapeutic agent since it may be inactivated by serumalbumin. This latter observation deserves further examinations.

(2) Studies on the possible role of ribonuclease (RNase) in host defense against TBE and other arboviruses.

Recently it has been reported that not only single-stranded viral RNS, but also the nucleocapsid of Semliki-Forest virus (SFV) can be degraded by RNase (15,16). Therefore it was of interest to determinewhether this enzyme, which is produced by some cells of the organism, contributes to the defense of the infected host against RNA containing viruses.

Serum as well as spleen and brain, which play an important role in the pathogenesis of arbovirus infections, were taken from noninfected Swiss Albino mice (10 g). The organs were homogenized with 1/15 M phosphate buffer of pH 7.8 and diluted 1:10 relative to their wet weight. Also the serum was diluted 1:10 with this buffer. The suspensions were assayed for RNase activity by the following method: A 1% solution of yeast RNA was incubated for 1 hour with the enzyme-containing material at pH 7.8 and at a temperature of 37°C. Thereafter the material was kept in an ice bath and high molecular RNA was precipitated by an HCl-alcohol mixture. After centrifugation for 10 min. at 2,500 rpm the enzyme-degraded low molecular oligonucleotides in the supernatant were assayed by their UV-absorbance at 260 mμ. As control, buffer without enzyme was also measured. Thus it was learned that spleen tissue contained 4 times more RNase than the serum, while no enzyme was found in the brain.

This could, perhaps, account for the differences in the virus production by the spleen and the brain of mice (weighing 10 g) after subcutaneous infection with SFV-virus. The result of this experiment is shown in Fig. 2. It will be seen that 2 hours after the infection the injected virus has reached the spleen. At 4 hours p.i. eclipse phase is demonstrable; thereafter new virus is rapidly produced by the cells, but does not exceed a titer of 10^4 (for babymice, i.c.) in the spleen. However in the brain, where no RNase was found, titers of the virus up to 10^7 LD_{50} were produced. During the course of infection no significant change of the enzyme levels in the serum and in the organs were found, even in moribund animals.

In preliminary experiments babymice infected with SFV were treated intracerebrally with commercially available pancreatic RNase as well as with RNase, which derived from mouse spleen. Concerning fatalities or time of survival this treatment did not have any influence.

It was found that the serum of babymice contained much less of the enzyme than that of adult animals. Therefore

we initially thought, that this phenomenon had something to do with the wellknown higher susceptibility of baby-mice for most of the arboviruses. But later on we learned that there was no difference in the content of the enzyme in the spleen between suckling and adult mice.

In the course of infection with some arboviruses of sub-adult Swiss Albino mice we found a slight change in the content of the enzyme in the serum; after infection with West Nile and Tahyna viruses, which produce a nonfatal disease, a slight increase of RNase was found. By contrast, infection with TBE virus, which invariably kills Swiss Albino mice, was accompanied by a decrease of the level of this enzyme in the serum. However, changes were only slight and could not be reproduced constantly.

In an other experiment two species of mice, white Swiss Albino, which are highly susceptible for TBE, and resistant mice of the Species <u>Mus musculus spicilegus</u> were tested for their enzyme content. Both species had about the same levels of RNase in spleen, brain and the serum, which were not strongly altered during infection with TBE. Therefore resistance of <u>Mus musculus spicilegus</u> to TBE cannot be attributed to the action of RNase.

Although a great deal of work was done by us, the somewhat conflicting results do not allow us to draw any definite conclusion. However, we feel further work ought to be done to clarify the importance of RNase in the defense against virus infection.

Summary:

In a series of experiments with Semliki Forest, West Nile, TBE and Tahyna viruses the role of RNase as a possible defense mechanism against viral infection in mice was investigated. So far, no conclusive evidence was obtained that this enzyme influences the outcome of disease.

(3) Formation of interferon in two species of mice after infection with TBE virus.

Reservoir animals of arboviruses after infection usually show high viremia but do not develop disease. The cause of this phenomenon is not known. In the present study we investigated the question of whether or not interferon could be responsible for this natural resistance.

Susceptible white Swiss Albino mice (strain GP, NIH, Bethesda) from the breeding colony of the Institute of Hygiene, Vienna, as well as resistant mice of the free living species Mus musculus spicilegus, which also were bred at the Institute of Hygiene, were infected s.c. with $10^7 LD_{50}$ (for GP mice) of TBE virus (strain Hypr). All mice weighed 10 g at the beginning of the test.

From the 1st to the 4th day after infection daily 4 mice out of each group were blod. From the 5h to the 8th day p.i. daily 4 animals of both groups were sacrificed. Their brains were removed and suspended as previously described (17). Serum samples and brain suspension were assayed for virus content by titration in babymice and for interferon by FINTER's dye uptake-method (18).

From Table 18 it can be seen that viremia and content of interferon in serum was very similar in both species of mice. In contrast there were marked differences in the brains. The susceptible white mice produced high levels of interferon and virus, while the respective titers in the mice who resist disease reached only very low values. Thus our results only confirm the wellknown fact that TBE virus is a potent inducer of interferon. No evidence was obtained that the absence of disease in Mus musculus spicilegus can be attributed to a protective effect of this antiviral protein.

(4) The protective effect of Tilorone Hydrochloride on experimental TBE in mice.

Introduction:

In previous papers (19,20,21) we could demonstrate that the interferon inducing compound Poly I:C is capable of preventing experimental TBE in mice. However, its high toxicity precludes the extensive use of this substance in man (23,27,28,29).

Recently MEYER et al. (21,23,24) reported about a new interferon inducing substance called Tilorone HCl 2,7-bis [2- (diethylamino) ethoxy] fluoren-9-one which can be given orally (Fig.3). It seems to be of lower toxicity than Poly I:C (28). The present study was carried out to assess the effect of this new compound against experimental TBE in mice. In addition, Tilorone was tested in combination with low doses of Poly I:C. This was done in the hope to achieve high antiviral protection with low toxicity.

Material and Methods:

Mice: The mice, strain GP (NIH,Bethesda), derived from the breeding colony of the Hygiene-Institut, University of Vienna.

Drugs: Poly I/C was purchased from the Miles Chem.Comp. Elkhart, Indiana, USA. Tilorone HCl was received from the Wm.S.Merell Company, Cincinnati, Ohio. Of both compounds suspensions in PBS were made.

Interferon Assay: Interferon in serum was assayed by the dye uptake-method of FINTER (18,26) using L-cells and VSV as the challenge virus.

Results:

Fifty mice were treated orally with Tilorone HCl (100 mg/kg mouse). Blood was taken from 5 mice 4,6,8,12,16,24,27, 36,48 and 72 hours thereafter and serum samples were assayed for interferon. As can be seen in Fig.4, interferon was first detected in serum 6 hours after treatment; a peak was reached after 16 hours and no interferon was found in the specimens taken after 48 hours or later.

A second group of 40 mice received Poly I:C intraperitoneally in a dose of 10 mg/kg mouse. Half an hour, 1,2, 4,9,13,21 and 45 hours thereafter blood samples were taken from 5 animals each and interferon was measured in the serum. Fig.4 shows that Poly I:C induced interferon very rapidly as after 1 hour the maximum titer was reached already. However, circulating interferon was essentially gone by 13 hours.

The third group consisting of 70 mice was first treated with Tilorone HCl (100 mg/kg mouse) and also with Poly I:C (10 mg/kg mouse) 27 hours thereafter. Interferon was assessed in serum samples which were taken from 5 mice each at 4,6,8,12,16,24,27.5,28,31,36,40,48 and 72 hours after Tilorone HCl application. Fig.4 shows that mice treated with both inducers developed levels of interferon which were only slightly higher than those obtained after treatment with Poly I:C alone.

Table 19 illustrates that Tilorone HCl in a dose of 200 mg/kg mouse can prevent fatal TBE in mice. From 50 treated mice only 16 died after infection with 100 LD_{50} of the virus, while all 50 control mice, which received no drug, succumbed.

After a lower dose of Tilorone HCl (50 mg/kg mouse) 90% of mice died after a survival time that was slightly longer than with those of the controls.

In 3 experiments Tilorone HCl and Poly I:C were combined in low doses. Tilorone HCl was given 24 hours prior to and Poly I:C 3 hours after infection. Control groups of mice received at the same time one of the two inducers alone or no treatment at all. The doses of the two drugs as well as doses of infection in the 3 experiments can be seen in Table 20.

Although combined application of the drugs had resulted in a slight increase of Interferon titer, it is obvious from Table 20 that nothing was gained as far as rate and time of survival was concerned.

Discussion:

MAYER and KRUEGER (24) have shown, that Tilorone HCl is a potent interferon inducer, thus protecting mice against various virus diseases. The highest degree of protection was achieved, when the drug was given 24 hours prior to infection (24). Therefore, we also used the compound at this time.

On the other hand we have demonstrated that Poly I:C is capable of preventing TBE in mice. The drug was active even when given 3 hours after infection (20,21). Because of this it was used at this time throughout all tests.

Levels of interferon measured at different times in the serum of mice (see Fig.4) indicate that Tilorone HCl is an inducer of the virus-like type while Poly I:C stimulates an interferon as it is formed by the cell after treatment with endotoxin.

Although the formation of interferon after the application of the two compounds seems to follow different routes there is very little enhancing effect when both drugs are administered to the same animal. This is apparent from both the level of interferon in the serum and the protection provided against infection with TBE virus.

However it is apparent from our study that Tilorone HCl is highly active against TBE virus <u>in vivo</u>. If this substance is released for treatment of man it is certainly worth testing.

Summary:

Tilorone HCl induced interferon in the serum of mice thus affording protection against fatal TBE when given 24 hours prior to infection and in a dose of 200 mg/kg mouse. After application of a lower dose (50 mg/kg mouse) mice succumbed to the infection but had a slightly longer survival time than untreated controls.

Tilorone HCl was also used in combination with Poly I:C. The latter was given 3 hours after infection i.e. at a time, when the Tilorone HCl-induced serum interferon already decreased. Thus, a second peak of interferon was demonstrated, which was only slightly higher than that in non-Tilorone HCl treated control mice. However, the combined application of low doses of both interferon inducers did not show any advantage over the single application as far as rates and time of survival of mice were concerned.

Studies on Patients

(1) Diagnostic studies.

These studies were first done in the usual manner with the hemagglutination-inhibition (HI) test and the Complement-fixation (CF) test. Since the beginning of 1971 we switched to the 2-ME test which is described in detail on page 14 .

It was stated in last year's Final Technical Report (2) that our estimate of 200-300 cases of TBE that occur each year in Austria probably is still too low. The results of our diagnostic studies done in 1970 proved that our assumption was correct: From January through December 1970, a total of 320 cases of TBE was diagnosed in our laboratory. The patients were hospitalized in the following Austrian provinces: Vienna 45, Burgenland 7, Lower Austria 56, Upper Austria 27, Carinthia 112, Styria 73; this is the highest number of cases ever recorded since the beginning of our studies. However, upon comparison with data obtained in previous years (see Table 21) it becomes apparent that 1970 was not such an exceptional year as far as cases diagnosed in Vienna, Burgenland, Lower Austria and Upper Austria were concerned. The only real difference in the number of cases derived from the fact that all hospitals in Carinthia and one large hospital in Styria (Graz) have started sending all their specimens to us. The data from 1971 thus far available indicate that also this year morbidity of TBE will be about the same as in 1970.

Figures 5-9 show the distribution of the endemic areas of TBE virus in 5 provinces according to the information obtained by means of questionnaires sent to the patients. Each dot on the map indicates an area where in 1970 or 1971 (until July) a patient acquired a tick whose bite was infective and was followed by disease. In addition, foci verified in 1970 or 1971 through virus isolation from ticks were also incorporated into the Figures. The maps further illustrate the magnitude of the public health problem that TBE presents in Austria.

In reading the maps one is, perhaps, struck by the distribution of TBE in some of the provinces. However, we are quite convinced that as a rule the results reported represent the actual distribution of the virus. Only in Styria sampling was inadequate due to the fact that we have connections with but one hospital. There TBE is much more frequent than it appears from the map and probably occurs

in the whole well wooded area south of the Mur and Mürz rivers. North of this line the Alps start and the valleys soon exceed altitudes beyond 1000 m. It is our experience that although Ixodes ricinus ticks are still found above this level they do not reach densities of population which are necessary for sustaining a virus cycle in nature.

(b) 42 frozen sera stemmed from patients who had TBE some years ago. The sera were drawn at least one year after the disease.

(c) 61 acute phase sera from patients who were hospitalized in spring of 1971 with the suspected diagnosis of TBE. The sera were not subjected to freezing before testing in the HI test.

Treatment of sera with 2-ME: Nonspecific inhibitors in sera were removed by either the kaolin or acetone-method. Thereafter, 0.9 ml of this serum were mixed with 0.1 ml of 0.5 M 2-ME and incubated for 1 hour at 37 °C. For control, distilled water was used instead of 2-ME. Immediately thereafter with both samples the HI test was done using 4 units of antigen and testing the sera until a dilution of 1:1280. A difference in HI titers of 2 steps was considered as significant.

Results:

From Table 17 it can be seen that upon testing of the frozen sera listed under (a) in about 32% of established cases the diagnosis could be confirmed by the 2 ME-method. With early sera of uncertain cases of TBE as revealed in the CF-test, the 2-ME test gave the diagnosis in 46%. No serum from the patients listed under (b) who had suffered from TBE at least a year ago, showed a decrease in titer after 2 ME-treatment.

In an experiment (listed in Table 23) the effect of different concentrations of 2-ME on the results of the test was studied. It was learned that the amount of 2-ME did not affect the results in concentrations from 0.04 to 0.09 M in the serum.

Also there was no difference between incubation of serum with antigen at + 4 °C overnight and incubation for 4 hours at room temperature.

By contrast, the amount of antigen used in the HI test was of great importance for the result of the 2-ME test and should not exceed 4 units of antigen. Thus, in a test with 16 antigen units not only the serum titers were lower but also the difference between the 2-ME treated and the control sample was less markend and often decreased below 2 titer steps.

Comparing the results of the 2-ME tests of the frozen sera from 1970 with those of the fresh sera from 1971, it became obvious, that freezing and thawing has deletorious effects on the IgM antibodies. Besides we found that kaolin removed some of the large molecular antibodies from

(2) Early diagnosis of TBE in the hemagglutination-inhibition test by treatment of serum with 2-Mercaptoethanol.

Introduction:

TBE in man usually is diagnosed by serological methods. Virus isolation is only possible during the first viremic phase of the disease or from brain after death. Until recently diagnosis of TBE was made in our laboratory in the following manner: Whenever the disease was suspected blood was drawn from the patient and the serum was tested in the hemagglutination-inhibition (HI) test. If positive, a second blood specimen was obtained 10-14 days later, and the paired sera were tested with the complement-fixation (CF) test. Although this method gave excellent results, it was still too slow and time-consuming to be of aid to the clinician.

After infection with TBE virus the primary immunological response consists of the formation of IgM antibodies (30,31), whereas antibodies of the IgG type are only produced later during disease.

In the study reported here it was found that diagnosis of TBE can rapidly be ensured by the detection of IgM antibodies in a single serum drawn soon after the onset of the disease.

For this purpose we used 2-Mercaptoethanol (2-ME) which is known to inactivate the biologic activity of IgM-antibodies, while immunoglobulin G is unaltered. Therefore, a decrease of antibody titer of serum by 2-ME treatment should indicate that the antibodies are mainly of the IgM type and that the serum was derived from a patient with a recent infection. Such a procedure has already successfully been used for the rapid diagnosis of rubella (32,33) and Japanese B encephalitis (34) virus infections.

Material and Methods:

Hemagglutination inhibition (HI) test: The test was done following the classical procedure described by CLARKE and CASALS (35).

Sera: (a) 204 frozen (-20°) sera, taken early after the onset of disease, derived from patients who in 1970 have been hospitalized with nonbacterial meningoencephalitis. In 182 of these cases TBE was diagnosed with the CF test while in 22 cases the result of the CF test was inconclusive.

the serum. Because of these findings removal of nonspecific inhibitors is now performed with the acetone-method (35). With this technique excellent results were achieved: So far 61 sera were obtained which contained HI antibodies. In 51 cases TBE could be diagnosed with the 2-ME test and only in 3 of the 54 proved cases of TBE diagnosis had to be established with the CF test. In the remaining 7 cases the results of both the 2-ME and the CF tests indicated that the antibodies were due to an earlier infection and had nothing to do with the present disease.

Discussion:

TBE is an important medical problem in Austria. Therefore exact and rapid diagnostic methods are needed. From our study it is obvious that the 2-ME test represents a great improvement in the laboratory diagnosis of TBE of man, because the disease can now rapidly be diagnosed.

According to our experience we propose the following diagnostic procedures:

(1) Serum should be obtained from the patient immediately after overt TBE is suspected and tested in the HI test for antibodies, in dilutions 1:10 and 1:20. If negative, TBE can be excluded. If positive:

(2) the ME-test is performed using acetone for the removal of unspecific inhibitors. During the peak incidence of TBE when at least 30% of the sera are positive, it pays to shorten the procedure by omission of the screening for antibodies and immediate performance of the 2-ME test. Since the antigen-serum mixtures are incubated overnight, diagnosis of TBE can thus be made within 24 hours. If no difference in titer is demonstrable with and without 2-ME treatment:

(3) a second serum sample must be required and tested together with the first one in the CF test.

However, this will be necessary in less than 10% of the cases.

Summary:

It is known that 2-Mercaptoethanol (2-ME) is capable of inactivating antibodies of the IgM-type which are formed early during the course of viral diseases. In the present study it was investigated whether this compound can be used for an early diagnosis of TBE. For this purpose, sera from patients with acute TBE and from persons who contracted the disease at least one year before the serum was drawn, were investigated in the hemagglutination-inhibition

test prior to and after treatment with a 0.05 M concentration of 2-ME. It could be demonstrated that 2-ME causes a drop in HI titer in sera of patients with acute TBE. Some technical data are given concerning this 2-ME test. Following the procedure suggested by us about 90% of TBE cases can rapidly be diagnosed with only one serum available from the acute phase of the disease.

(3) Interferon in the CNS of fatal cases of TBE.

A study was undertaken to investigate the role of interferon in fatal cases of different viral encephalitides. So far materials from 5 cases could be obtained, of which 3 were TBE. This once more shows the fact that TBE is an important medical problem in Austria. The data, which were available about the patients, are summarized in Table 24.

Brains were prepared and suspended as described earlier for mouse brain (17). The suspensions were tested for interferon by the dye uptake-method of FINTER (18), which is now routinely used for interferon assay in our laboratory.

The results are listed in Table 25. Interferon was found in two cases of TBE, in which the disease took a very fast course. In one of those fatalities also virus could be isolated from the brain. The failure to isolate the virus in the second case probably was due to the fact that transport facilities were inadequate and the brain arrived at our laboratory in a rather autolytic state.

The third brain was devoid of both interferon and virus. In this particular case the patient had lived for more than 3 weeks after the onset of overt TBE.

The results of our study clearly indicate that interferon can be demonstrated in the CNS of patients who succumb to TBE, provided that patients die early in the course of disease. Thus it seems evident that in TBE interferon production is not the defense mechanism, on which death or survival depends.

(4) Immunoglobulin.

Since 1970 human Gammaglobulin against TBE virus is available for prophylactic use in man. Details were given in last year's Final Technical Report (2). This year (1971) quite a run has started on the drug which soon proved to be in short supply. Against our advice the manufacturers produced too small quantities. Thus far about 3,000 doses were sold but according to the producer, the demand is about 10 times higher. Only time can tell to which extent the immunoglobulin is able to prevent or modify TBE. The only thing we do know is, that none of this and last year's patients had received the drug as prophylactic prior to becoming ill. The demand for TBE immunoglobulin which went far beyond the expectations shows that great efforts must be made to supply our population with a safe and potent vaccine. This will be one of our major tasks in the coming years.

M o s q u i t o b o r n e v i r u s e s
i n A u s t r i a

(1) Introduction

During the pest yeare we have cerried out exteneive field end laboratory studiee on the two moequito-borne viruees eo fer isoleted in Auetria, the Tahyne virue and the Calovo virue. Theee investigatione yielded meny reeults on the ecology and biology of the two viruses which are summerized in the Final Technical Reports 1969 and 1970 (36,2). Some important features in the ecology of both viruses remained, however, uncleer. One of the most striking problems is the mode of hibernation and the periodicity of the occurrence of the two viruees as well ee the apperently very limited ereas in which Tahyne end Celovo viruees ere endemic.

In order to get further information on the ecology of theee arboviruses the following investigations were cerried out:

(1) Virus isolation experimente from moequitoes collected in the Eastern part of the Neusiedlersee eree in 1968. (Most of the results of theee etudies which have been finiehed meanwhile were already dealt with in the Final Technical Report 1970(2)).

(2) Virus isolation experimente from mosquitoes collected in the Western pert of the Neusiedlersee in 1970.

(3) Virus isolation experiments from overwintering mosquitoes collected in the Eastern part of the Neusiedlersee during spring 1971.

(4) Exposure of indicator rabbits in the Western pert of the Neusiedlersee in 1970.

(5) Serological survey with bovine eere.

(6) Virus isolation experiments and serological survey with avian sera.

(7) Experimental infection of hare with Calovo virus.

(2) Collections of mosquitoes and virus isolation experiments in the Eastern part of the lake in 1968.

In 1968, collections of mosquitoes were carried out in the Eastern part of the Neusiedlersee area near the village Apetlon (Seewinkel) in a cow barn during the day and in the field by sweeping entomological nets through the air from evening twilight until one hour after sunset. With the exception of those mosquitoes collected on September 3 all specimens had been already identified when the Final Technical Report 1970 was written; there the results are demonstrated in tables.

On September 3, the following species were found (in brackets number of specimens collected/number of specimens tested for virus/number of pools/number of virus strains isolated):

Anopheles maculipennis	(7/7/2/-)
Anopheles claviger	(2/2/2/-)
Mansonia richiardii	(703/703/7/-)
Uranotaenia unguiculata	(2/2/2/-)
Culiseta annulata	(7/7/4/-)
Culex pipiens	(50/50/4/-)
Culex modestus	(57/57/6/-)
Aedes flavescens	(18/18/3/-)
Aedes cantans	(10/10/3/-)
Aedes caspius and Aed. dorsalis	(1464/1464/30/3 strains of Tahyna virus)
Aedes vexans	(9/9/3/-)

Altogether 10,544 mosquitoes belonging to 12 species were collected in the cowbarn; from these 10,564 were tested in 272 pools for virus, whereby one strain of Tahyna virus (deriving from a pool of Anopheles maculipennis collected on July 10) and two strains of Calovo virus (deriving from two pools of Anopheles maculipennis collected on August 17) were isolated.

The mosquitoes collected outdoors comprised 16,250 specimens belonging to 12 species; from these 16,242 specimens were tested for virus in 446 pools, whereby 10 strains of Tahyna virus were isolated. All strains derived from pools of Aedes caspius and Aedes dorsalis collected on June 25 (4 strains), on August 17 (2 strains), on September 3 (3 strains) and on September 17 (1 strain).

The population dynamics of mosquitoes collected outdoors and in the cowbarn and the relativo abundance of the six most frequent species are shown in Fig. 10 and 11. Those days on which mosquitoes infected with virus were collected are indicated by a T (=Tahyna virus) respectively by a C (=Calovo virus).

(3) Collection of mosquitoes attacking men and virus isolation experiments in the Western part of the lake in 1970.

The Neusiedlersee area is one of the touristically most important parts in Austria. This is particularly the case in the Western part of the lake where large parts are suitable for all kinds of water sports. Additionally, in Mörbisch, a village at the Hungarian border, festivals take place at the shore of the lake in open air theatre every year during the summer months. Thus, masses of mosquitoes are attracted, heavily attacking man, and representing a touristical problem.

In August 1970, we therefore carried out preliminary studies on the mosquitoes attacking man at the Western side of the lake.

On 4 days during the period from August 8 to August 19 altogether 890 mosquitoes were collected in 5 localities in the surroundings of Rust and Mörbisch (see Fig. 12), the two touristically most important villages. Only those mosquitoes were caught which were attacking one of the three persons acting as bait. They were sucked from the skin by aspirators and then frozen in dry ice.

The results of the collections are shown in Fig. 13-17. From the diagrams the fluctuations of activity of the species observed during the period from late afternoon until night can be seen. From the 890 females collected, 799 (=89.8%) specimens were represented by Culex modestus, 66 (=7.4%) by Mansonia richiardii and 25 (=2.8%) by Anopheles maculipennis. It will be seen that Culex modestus was active during sunshine in the afternoon as well as after sunset. Mansonia richiardii showed two peaks of activity: a small one during late afternoon and a large one after sunset. The first peak may be explained by the reduction of light intensity and by the displacement of sunlight into the long wave spectrum when the sun immerses into the haze stratum above the horizon on late afternoon which is being "misunderstood" by the mosquito whose actual activity depends on the darkness. The activity then declines as a result of habituation to the more or less constant light intensity and rises then rapidly after sunset.

The mosquitoes were tested for virus in 49 pools by i.c.infection of baby mice. No virus was isolated.

(4) Collections of overwintering mosquitoes and virus isolation experiments in the Eastern part of the lake during spring 1971.

CHIPPAUX et al. (37) reported the isolation of a strain of Tahyna virus from females of *Culex modestus* which were collected in the south of France in December. As *Culex modestus* overwinters in the imaginal stage the question arose whether the virus may hibernate in the mosquito-species. *Culex modestus* occurs in all parts of the Neusiedlersee area; so far it was, however, not possible to isolate the virus from this species.

In order to study the possibility of overwintering of Tahyna virus in *Culex modestus* it was intended to carry out collections of this species and virus isolation experiments during spring. For this purpose, a large case (about 4 m^2 and 2 m high) surrounded by a fence was placed at the Sandeck, an area situated at the margin of the reed zone in the Eastern part of the Neusiedlersee area near the village Illmitz where the Tahyna virus had often been isolated during the past years. On March 18, three hens were put into the cage which was easily accessible for mosquitoes. Sentinel hens were chosen as *Culex modestus* is an ornithophilic species although feeding on mammals also. Parts of the cage consisted of reed blocks where mosquitoes, particularly *Culex modestus*, like to rest.

On March 21,24,26,30, April 2,6,10,17,18,20,22,29, May 3, 8,12,13,17,20,21,23,26,29 and June 2 the cage was inspected and resting mosquitoes were collected and immediately frozen in dry ice. The experiment was finished on June 2, as at this time the overwhintering generation of *Culex modestus* had certainly died out.

The results of collections of mosquitoes are shown in Table 26; it will be seen that only few specimens of *Culex modestus*, namely 14 females, and 102 females of *Anopheles maculipennis* were caught. It is remarkable that *Culex modestus* was found until the middle of April only. The mosquitoes were pooled and tested for virus in baby mice in the usual manner. No virus was isolated.

(5). Exposure of indicator rabbits in the Western part of the lake in 1970.

In 1969, virus isolation experiments and serological studies with sentinel rabbits had been carried out in the Western part of the Neusiedlersee which led to the assumption that the Tahyna and Calovo viruses might not occur in that part of the Neusiedlersee area because neither any virus strain nor antibodies against Tahyna or Calovo virus could be detected.

For further information 4 indicator rabbits were exposed on a platform in the Western part of the lake from June 15 until October 10. This is the period during which Tahyna and Calovo viruses were regularly isolated in the Eastern part of the lake. The blood samples taken on October 10 were tested in the NT for antibodies against Tahyna and Calovo viruses.

All rabbits proved to be serologically negative against both viruses.

(6) Serological survey of bovine sera.

Many of the virus strains isolated in the Neusiedlersee area during the past years derived from mosquitoes which had been collected in cowbarns (38,39 and this Report).

It was,therefore, of interest to study the question whether and to which extent cattle are involved in the virus circulation.

For this purpose, 641 bovine blood samples were collected in 5 communities situated in the Eastern Neusiedlersee area, namely from Frauenkirchen (77), Tadten (123), Wallern (110), Pamhagen (143) and Illmitz (188). All cattle were at least one year old.

The sera were tested for neutralizing antibodies against Tahyna and Calovo viruses in a tissue culture of the cell line GMK-AH-1 with methods previously described (1).

The results are shown in Tables 27 and 28. It will be noted that 4% to 20.7% (average: 11.7%) of the cattle had antibodies against Tahyna virus and 57.1% to 78.7% (average: 68.3%) had antibodies against Calovo virus.

(7) Survey with avian sera.

In connection with the question whether the ornithophilic mosquito-species Culex modestus might be involved in the circulation of Tahyna virus (38), we started a program for collections of sera of starlings (Sturnus vulgaris) during late summer 1970; this bird species is very frequent in the area under investigation. In addition, it was intended to get information on the possible role of starlings for the incidence of other arboviruses. During the first weeks we were confronted with many technical and juridical problems, which could, however, finally be solved.

For capturing starlings, Japanese nets measuring 6 x 2 m were used which were put up in several parts of the Western Neusiedlersee area.

Altogether 32 starlings were caught and bled for virus isolation experiments and for serology.

All blood samples were injected i.c. into baby mice. From none of the samples virus could be isolated.

So far, only 23 sera were tested in the NT (for method see Annual Report 1969) for antibodies against Tahyna virus, whereby two samples proved to be positive.

The sera will be tested in the HI test for antibodies against other arboviruses also.

(8) Experimental infection of hare (*Lepus europaeus* L.) with Calovo virus.

Extensive serological investigations have shown that hares have antibodies against Tahyna virus to a high extent and that they represent an important factor in the circulation of this virus. It was, however, surprising, that none of 268 hares shot in the endemic area in the Eastern Neusiedlersee area had antibodies against Calovo virus (40). Thus, the question arose whether hares are oven suceptible for the Calovo virus, a question which was of particular interest, as the rabbit regularly responds to the infection with Calovo virus by forming neutralizing antibodies; the rabbit is indeed an excellent indicator for the occurrence of that virus (41).

In order to clarify the question, 5 three months old hares were infected subcutaneously with an extraneural strain of Calovo virus (a homogenate of 20 supernatants of pools of *Anopheles maculipennis* from which the virus had been isolated). Two hares (Nr.1 and Nr.2) were infected each with 100,000 LD_{50}, two (Nr.3 and Nr.4) with 10,000 LD_{50}, and one hare (Nr.5) with 1,000 LD_{50}/baby mice. Blood was taken on the 1st, 2nd, 3rd, 4th, 5th and 6th day p.i. and injected i.c. into baby mice undiluted and in three dilutions (10^{-1}, 10^{-2}, 10^{-3}).

Viremia was not detectable in any of the five hares. Four weeks p.i. blood was taken again from all animals and tested for neutralizing antibodies against Calovo virus (method see (35)).In none of the hares neutralizing antibodies could be detected.

Five weeks p.i. the hares Nr.1 and Nr.5 were each infected with 5,000,000 LD_{50}/baby mice of the neuroadapted strain CUL 16345 of Calovo virus (6th passage in baby mice). Four weeks later, blood was taken for serology. None of the two hares showed a conversion of antibodies against Calovo virus.

From this it appears that hares are - in contrast to rabbits - not susceptible for the Calovo virus.

Discussion

The results of investigations reported above have confirmed in several details two observations already made in prior studies:

(1) The Tahyna virus and the Calovo virus regularly occur during a very limited period of the year in the Eastern part of the Neusiedlersee.

(2) So far, we have no evidence of the occurrence of either of the two viruses in the Western part of the lake.

In the Eastern part of the lake field investigations were carried out from 1965 onwards. In the Western part from 1969 onwards.

In the years 1966 until 1970 altogether 91 sentinel rabbits were exposed in different parts of both sides of the lake mostly from April until November. The majority of the rabbits exposed in the Eastern part of the Neusiedlersee area developed antibodies against Tahyna and Calovo viruses during certain short periods (see Fig.18 and 19). Correlating those dates with those obtained from investigations on mosquitoes (38,39,41 and this Report) one arrives at the following conclusions:

(1) So far in the endemic area at the Neusiedlersee (as well as in other parts of Central Europe) there is no evidence of the circulation of Tahyna virus before June 10 and after September 17, and of Calovo virus before July 8 and after October 1.

(2) The actual period of evident circulation of the two viruses is, however, in most years much shorter (in some years apparently even a few days only) and covers different parts of the periods mentioned under (36).

(3) The appearance of neutralizing antibodies against Tahyna and Calovo viruses largely coincides with the appearance of mosquitoes infected with these viruses. From this it may be concluded that other blood sucking Diptera attacking rabbits do not act as vectors.

The reasons for regular evidence in the Eastern Neusiedlersee area but failure to isolate both viruses in the Western part can be explained by the much less favourable ecological conditions for the virus circulation in the West of the area under investigation.

As regards the Tahyna virus there is no doubt that Aedes caspius and Aedes dorsalis are the main arthropod hosts while hares represent the main vertebrate host. Both occur in lower population densities in the west; this is particularly the case with Aedes caspius and Aedes dorsalis which cannot develop in the lake but need intermittent waters which dry out at certain periods during which the eggs are laid upon the dry soil. The Seewinkel comprises numerous waters of this kind, on the Western part there are, however, practically no intermittent waters.

As regards the Calovo virus it can now be concluded that cattle are the main vertebrate host (at least in the Neusiedlersee area). From 641 cattle sera tested 438 (=68.3%) had antibodies against Calovo virus, while only 75 (=11.7%) were positive against Tahyna virus. So far, only one serological survey including Calovo and Tahyna viruses has been

carried out with cattle, out of 93 cattle from Slovakia 6.4% had HI antibodies against Tahyna virus and 12.9% against Calovo virus (42).

The results reported here show that cattle represent a good indicator for the occurrence of Tahyna virus. However, this domestic animal does not appear to participate in the natural cycle of the virus.

In case of the Calovo virus the situation is quite different. Among the vertebrate species other than cattle occurring in Central Europe only roe deer and horses may have a certain importance for the cycle of the Calovo virus (40). In the endemic east to the Neusiedlersee (and probably in most other parts of Europe also) the population densities of both species are, however, probably too low for the maintenance of the virus cycle. The high antibody-rates against Calovo virus found in cattle in the Eastern Neusiedlersee area lead to the suggestion that the virus cycle depends on a sufficient number of cattle and a certain way of keeping cattle. It will be necessary to confirm this assumption by quantitative ecological studies; in particular it will be of interest to determine the height and duration of viremia in cattle.

Two facts, however, confirm the concept of the essential role of cattle, namely the fact that most of all Calovo virus strains isolated during the past years derived from Anopheles maculipennis collected in cowbarns on one hand and the special kind of keeping cattle that is common in the Eastern Neusiedlersee area. In that area, the Seewinkel, about 10,000 cattle are kept among which one third are individuals which have been born outside the area and are held for fattening only. These cattle are imported from other parts of Austria (mainly from Upper Austria and from Styria) mostly at an age of a few weeks and kept in the Seewinkel for about 1 1/2 years. Those cattle which are imported at an age of some months may stay only a few months (longest period: September until June of next year) and are then sold. Thus the virus reservoir is permanently replaced so that every year a sufficiently high number of susceptible and non immune cattle are introduced into the virus cycle. In addition, cattle are the most important hosts of Anopheles maculipennis messeae, thus contributing to the annual mass development of that mosquito-species. Fluctuations in the intensity and period of virus circulation in different years may be traced back to fluctuations of number and age structure of the cattle imported and to the dates of import.

This is a remarkable example of human influence on the ecological system of an arbovirus.

It is of interest that in India high antibody-rates against Chitoor virus (which is apparently indentical with Calovo virus) were detected in different domestic animals (goats, sheep, cattle, buffaloes, camels, horses and donkeys). (43,44). Thus it becomes evident that the spectrum of vertebrate hosts of Calovo virus is mainly represented by domestic animals.

It is a little surprising that the hare (Lepus europaeus) does not develop viremia nor antibodies after experimental infection with Calovo virus, while rabbits regularly show an immunological response. In previous experiments it could be shown that foxes (Vulpes vulpes) are susceptible to the virus, while badgers (Meles meles) develop neither viremia nor antibodies. Summarizing these results one arrives at the conclusion that the Calovo virus has an extraordinarily limited spectrum of vertebrate host species.

The most important problem which still remains to be solved in the ecology of the mosquito-borne viruses occurring in Central Europe is the mode of hibernation; many experiments have been carried out during the past years in our institute and by scientists in Czechoslovakia which have shown several possibilities of overwintering (36,2). Recently the Tahyna virus has been isolated from females of Culex modestus collected in December in the south of France (37). The authors suggest that the virus overwinters in Culex modestus. In our field studies carried out during spring 1971 not more than 14 hibernating females of Culex modestus could be collected although a method was used which is - according to our experience - selective for this species. From this result it must be concluded that it is very rare in spring - at least in the Eastern part of the lake. Most specimens of Culex modestus collected during other seasons (mainly late summer) were indeed caught in the Western part. As pointed out above, however, so far we have no hint of the occurrence of the virus in the west, while we have isolated many strains at that point (Sandock) where the collections of mosquitoes were done in spring 1971. So far, we have not any evidence for the hibernation of Tahyna virus in Culex modestus in Austria. It is conceivable that overwintering of the virus is possible in this mosquito but suggest that this mode of hibernation is not the actual one in the virus cycle. It will be necessary to study this question carefully in detail so that definite conclusions become available.

In connection with this problem the preliminary studies on avian sera were carried out. The finding of two sera of starlings with neutralizing antibodies against Tahyna virus is of

interest and gives a hint for a possible participation of ornithophilic mosquitoes (e.g. *Culex modestus*) in the circulation of the virus. Also this question will, however, require further studies. A larger survey with avian blood samples is intended. We are trying to get permission for catching and bleeding all bird species occurring in the Neusiedlersee area. We plan to test all blood samples for virus and for antibodies against a selected number of arboviruses. In addition, it will be necessary to do some experimental infections of birds (one to three species) with Tahyna virus in order to get exact data on susceptibility, viremia and antibody formation.

Summary

(1) In 1958, collections of mosquitoes for virus isolation experiments were carried out in the surrounding of the village Apetlon in the Eastern Neusiedlersee area from April to October. Altogether 26,815 specimens belonging to 13 species were collected from which 26,006 were tested in 718 pools for virus. Two strains of Calovo virus were isolated from *Anopheles maculipennis*, 10 strains of Tahyna virus derived from mixed pools of *Aedes caspius* and *Aedes dorsalis* and one strain of Tahyna virus was isolated from *Anopheles maculipennis*.

(2) During 4 days in August 1970 mosquitoes attacking man were collected by sucking them from the skin near the touristically important villages Rust and Mörbisch in the Western part of the lake. Altogether 890 specimens were collected, namely 799 *Culex modestus*, 66 *Mansonia richiardii* and 25 *Anopheles maculipennis*, and tested for virus. No virus could be isolated. The dynamics of activity of the mosquitoes from late afternoon until night is shown in diagrams. It is of interest that *Mansonia richiardii* which is active nearly exclusively after sunset has a small first peak of activity in the late afternoon when the sun immerses into the haze stratum above the horizon. Probably the sudden decline of light intensity and the displacement of light into the long wave spectrum is temporarely "misunderstood" by the mosquito.

(3) In spring 1971, mosquitoes for virus isolation experiments were collected at Sandeck in the Eastern part of the Neusiedlersee area using sentinel hens as baits. Altogether 116 mosquitoes could be collected during the period from March 21 to June 2, namely 14 females of *Culex modestus* and 102 females of *Anopheles maculipennis*. No virus could be isolated from these mosquitoes. It is suggested that the Tahyna virus may occasionally overwinter in

Culex modestus, but does it probably not as a rule, as the mosquito is apparently too rare.

(4) From June until October 1970, 4 indicator rabbits were exposed in the western part of the lake near Rust. None of the animals developed antibodies against Tahyna and Calovo viruses. So far, there is no hint for the activity of one of the two viruses in the Western Neusiedlersee area. This can be explained by the striking difference in the ecological conditions between the two sides of the lake.

(5) From five communities situated in the Eastern Neusiedlersee area 641 cattle sera were tested for antibodies against Tahyna virus and Calovo virus. In 75 sera (=11.7%) antibodies against Tahyna virus were found, 436 (=68.3% were positive against Calovo virus. From this it appears that cattle are not important hosts but good indicators for the Tahyna virus. In case of the Calovo virus cattle are, however, apparently the essential vertebrate host and virus circulation seems to depend on a sufficient number and certain age structure of cattle, since it is found in the Eastern (but not in the Western) Neusiedlersee area.

(6) During late summer 1970, 32 starlings (*Sturnus vulgaris*) were caught in the Neusiedlersee area. Blood was taken for virus isolation and serology. From none of the 32 samples tested was virus isolated. From 23 sera tested two proved to be positive against Tahyna virus. Thus it seems possible that birds and ornithophilic mosquitoes take part in the circulation of the virus.

(7) Five hares (*Lepus europaeus*) were s.c. infected with variable doses of Calovo virus. None of the animals developed viremia nor antibodies against the agent. From this it appears that hares are - in contrast to rabbits - not susceptible to the Calovo virus. It becomes evident that this virus has a very limited range of vertebrate hosts, preferably domestic ungulates.

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Table 1a: Number of ticks (*Ixodes ricinus*) collected 1970 in different areas in Lower Austria and virus strains isolated therefrom.

Excursion date	Number of: nymphs collected	(pools)	strains isolated	adults collected	(pools)	strains isolated
Gfi[illegible]oer						
June 17-20	141	(29)	-	25	(11)	-
Sept. 20-21	149	(17)	-	14	(3)	-
Strelzhof/Willendorf						
June 20-21	289	(17)	1	60	(15)	1
	1*	(1)	-			
Sept. 28-29	479	(25)	-	17	(9)	-
Hornstein						
June 6-7	1014	(50)	-	104	(32)	-
	1*	(1)	-			
Sept. 24-25	235	(27)	-	13	(6)	-

* Haemaphysalis concinna

Table 1b: Number of ticks (Ixodes ricinus) collected 1970 in different areas in Lower Austria and virus strains isolated therefrom.

Virus strain No.	Pool size	Location	Control point
30766	23 nymphs	Strelzhof/ Willendorf	11
30767	3 males	-"-	11

Table 2: Number of ticks (Ixodes ricinus) collected 1971 in different areas in Lower Austria and virus strains isolated therefrom.

Excursion date	Number of nymphs collected	(pools)	strains isolated	adults collected	(pools)	strains isolated
Bernstein II						
April 9	305	(15)	-	21	(11)	-
Bernstein II						
April 9	64	(4)	-	4	(2)	-
Hohenegg I						
April 11	122	(6)	-	13	(3)	-
May 22	438	(22)	-	12	(2)	-
Hohenegg II						
April 1[illegible]	103	(5)	-	5	([illegible])	-
May 23	227	(12)	-	26	(5)	1
Schottwien						
April 12	95	(9)	-	29	(9)	-
Grafenbach						
May 15-16	506	(25)	-	26	(5)	-
Platz						
May 15-16	125	(6)	-	18	(5)	-
Neudörfl						
June 16	212	(11)	-	20	(14)	1
Wiltendorf						
June 24	283	(14)	2	21	(4)	-

Table 3: Results of tick collections before and after Gardona treatment in Hernstein I.

	Field No.	Sept.24 Number of nymphs	(adults)	Sept.25 Number of nymphs	(adults)	Oct.6 Number of nymphs	(adults)
Treated fields	2	15		3		4	
	3	6		1		-	
	4	6		1		1	
	7	15		2		2	
	8	6		3		5	(2)
	12	11		1		-	
	16	5		5		-	
		64		16		12	(2)
Untreated fields	1	7		2		4	(1)
	5	13	(3)	2		14	(2)
	6	4		2		12	
	9	3		6		4	
	10	8		5		8	(1)
	11	5		3		7	(1)
	13	1		3		3	
	14	3		4		8	
	15	3	(2)	6	(1)	3	
		47	(5)	33	(1)	63	(5)

Table 4: Results of tick collections before and after Gardena treatment in Hernstein II and Mühileiten.

Hernstein II		April 27		April 30		May 5	
	Field No.	Number of nymphs	(adults)	Number of nymphs	(adults)	Number of nymphs	(adults)
Treated fields	1	19		1	(1)	1	(1)
	3	7		1		3	(2)
	5	21	(2)	4	(2)	7	
	7	21	(2)	2		2	
		68	(4)	8	(3)	13	(3)
Untreated fields	2	12	(4)	12		20	
	4	12		4		7	
	6	18	(2)	9	(4)	14	(1)
	8	16		6	(2)	17	(1)
		58	(6)	31	(6)	58	(2)
Mühlleiten		May 7		May 10		May 15	
Treated fields	1	17	(1)	-		-	
	3	23		1		-	
	5	5	(2)	-		-	
	7	14	(2)	1		-	
		59	(5)	2		-	
Untreated fields	2	4	(2)	8		3	
	4	14	(1)	6		3	(1)
	6	12	(2)	8	(3)	3	
	8	5	(2)	4	(2)	2	
		35	(7)	26	(5)	11	(1)

Table 5: Results of small mammal trapping in Taggenbrunn.

Excursion No.	Date	Trap units	Trapped animals
1	May 26-28 (1970)	460	1 Clethrionomys glareolus
2	Sept. 15-17 (1970)	360	2 Apodemus spec. (juveniles) 2 Clethrionomys glareolus
3	Nov. 8-10 (1970)	260	7 Apodemus spec. (subadults) 6 Clethrionomys glareolus 1 Microtus arvalis
4	April 18-19 (1971)	260	none

Table 6: Yield of final steps in the preparation of receptor substance for arboviruses (TPI-Ca).

Starting material (g monkey brain)	mg raw TPI-Ca	mg purified TPI-Ca	Yield per cent
300	118	85	72
200	88	66	75
300	102	87	73
300	119	95	80
300	104	82	79
300	130	91	70
300	108	87	80
300	107	80	75
400	144	120	83
300	147	109	74
300	102	86	86
Sum:3,300 g	1,269 mg	988 mg	

Table 7: Smallest amount of TPI-Ca still inhibiting the HA of 4 HAU of TBE virus at different pH values. (Incubation for 15 min. at 0°C).

pH	µg TPI-Ca
6.0	0.04
6.5	0.04
7.0	0.08
7.4	0.08
7.9	0.15
8.5	0.3
9.0	0.6

Table 8: Smallest amount of TPI-Ca still inhibiting the HA of 4 HAU of TBE virus at different temperatures (Incubation for 15 min. at pH 7.4).

Temperature	µg TPI-Ca
0°C	0.08 µg
22°C	0.3 µg
37°C	0.6 µg

Table 9: Smallest amount of TPI-Ca still inhibiting the HA of 4 HAU of TBE virus at different periods (Incubation at pH 7.4 and 0°C).

Time	µg TPI-Ca
1 min.	0.04 µg
2 min.	0.04 µg
4 min.	0.08 µg
8 min.	0.08 µg
15 min.	0.15 µg

Table 10: Titer of TBE virus preparations after incubation for 3 min. at pH 7.4 and 0°C with different amounts of RS, followed by 10 min.incubation with basic substance under the same conditions.

	Exp.1 Strepto-mycin	Exp.2 Strepto-mycin	Exp.3 Salmin	Exp.4 Strepto-mycin
Virus + 12 µg RS	2^3	n.d.	n.d.	n.d.
Virus + 12 µg RS + base	2^9	n.d.	n.d.	n.d.
Virus + 25 µg RS	2^2	2	2	2
Virus + 25 µg RS + base	2^7	2^5	2^7	2^6
Virus + 50 µg RS	2	2	2	n.d.
Virus + 50 µg RS + base	2^8	2^4	2^6	n.d.
Virus + buffer (control)	2^8	2^7	2^9	2^7
Virus + base (control)	2^8	2^7	2^9	2^8

Table 11: HA-Titration (1:1 dilution series) at heat inactivated TBE virus after combination with RS at pH 7.4 for different periods and at different temperatures, followed by separation from the RS by addition of streptomycin for 10 min. at 0°C and pH 7.4.

Temp.	Time	HA pattern							
0°C	2 min.	+	+	+	+	+	-	-	-
	4 min.	+	+	+	+	(+)	-	-	-
	8 min.	+	+	+	+	(+)	-	-	-
	16 min.	+	+	+	+	(+)	-	-	-
37° C	5 min.	+	±	-	-	-	-	-	-
	10 min.	±	-	-	-	-	-	-	-

Table 12: Per cent of infective TBE virus firmly bound to purified RS (not separable by streptomycin) after different periods of reaction at pH 7.4 and 0°C (Values from a graphic interpolation of actually observed HA titers).

Time	Strongly bound virus
0 sec.	0 %
10 sec.	81 %
20 sec.	92 %
40 sec.	95 %
80 sec.	96 %
150 sec.	96 %
300 sec.	97 %
600 sec.	98 %

Table 13: Influence of TPI on infectivity of TBE and Dengue viruses assayed in mice.

Virus	Dose of virus (LD_{50})	Amount of TPI	pH	Inhibition (LD_{50})	%
TBE	$10^{2.5}$	8 µg	6.4	$10^{1.0}$	90
TBE	$10^{1.8}$	8 µg	7.0	$10^{1.2}$	>90
TBE	$10^{2.5}$	8 µg	7.3	$10^{1.0}$	90
TBE	$10^{3.4}$	8 µg	7.5	$10^{2.3}$	>99
TBE	$10^{2.5}$	1.6 µg	7.5	$10^{0.7}$	<90
TBE	$10^{0.9}$	8 µg	9.0	0	0
TBE	$10^{0.6}$	8 µg	9.0	0	0
Dengue II	$10^{1.0}$	8 µg	7.0	$10^{1.0}$	90
Dengue II	$10^{1.3}$	8 µg	7.5	$10^{1.3}$	>90

Table 14: Influence of TPI on infectivity of Semliki and Sindbis viruses assayed in mice.

Virus	Dose of virus (LD_{50})	Amount of TPI	pH	Inhibition (LD_{50})	%
Semliki	$10^{3.1}$	8 µg	6.0	0	
Semliki	$10^{3.1}$	8 µg	6.4	0	
Semliki	$10^{3.0}$	8 µg	7.0	$10^{0.4}$	<90
Semliki	$10^{2.9}$	8 µg	7.5	$10^{0.2}$	<90
Sindbis	$10^{2.5}$	8 µg	6.4	0	0
Sindbis	$10^{2.6}$	8 µg	6.7	$10^{0.1}$	<90
Sindbis	$10^{2.7}$	8 µg	7.5	$10^{0.5}$	<90
Sindbis	$10^{2.2}$	8 µg	7.5	0	0

<u>Table 15:</u> CF-test of anti TPI and control sera with several antigens.

Antigen		Serum		
		Anti-TPI	Anticerebroside	Control
Mouse brain suspension		1 : 80	1 : 320 ±	1 : 10
Myelin		1 : 80	1 : 320	1 : 10
TPI acid		1 : 10	0	0
TPI sodium		1 : 10	0	0
TPI-lecithin	1:50	1 : 20 ±	0	0
	1:1	1 : 40	0	0
	27:1	1 : 40	-	-

Table 16. Summary of neutralization-inhibition tests with anti-TPI and control sera.

Dose of virus	TPI	Serum: Anti-TPI	Serum: Anticerebroside	Serum: control	Inhibition of neutralization
3000 LD_{50}	0.4 mg/ml	undiluted	-	-	0
300 LD_{50}	0.4 mg/ml	1 : 5	-	-	50 %
300 LD_{50}	0.4 mg/ml	-	1.5	-	50 %
300 LD_{50}	0.4 mg/ml	-	-	1.5	90 %

Table 17: Summary of neutralization-inhibition tests with sephadex-fractions of anti-TPI and control sera.

Dose of virus	TPI	Fraction of						Inhibition of Neutralization
		Control serum			Anti-TPI-serum			
		IgM	IgG	Alb	IgM	IgG	Alb	
100 LD_{50}	0.4 mg/ml	1:5	-	-	-	-	-	<90 %
		-	1:5	-	-	-	-	90 %
		-	-	1:5	-	-	-	99 %
		-	-	-	1:5	-	-	90 %
		-	-	-	-	1:5	-	90 %
		-	-	-	-	-	1:5	99 %

Table 18: Interferon in the serum and brain of mice susceptible and resistant to TBE.

	Days p.i.	Swiss albino (susceptible)		Mus. musc. spic. (resistant)	
		Virus	Interferon	Virus	Interferon
Serum	1	3.6[1]	160[2]	3.0	160
	2	3.7	320	6.3	320
	3	4.3	320	3.5	320
	4	2.8	160	1.7	160
Brain	5	4.0	640	3.2	160
	6	7.0	1280	3.0	320
	7	6.7	1280	2.8	320
	8	7.4	2550	2.7	320

[1]: log to the basis 10; LD_{50} assayed in suckling mice in 0.012 ml.

[2]: reciprocal value of titer

Table 19: The effect of Tilorone HCl on experimental TBE virus infection (100 LD_{50}) in mice.

Dose and time of treatment	Number of mice infected	Number of fatalities	Average survival time
200 mg/kg mouse 24 hrs. p.inf.orally	50	16	12.2 days
50 mg/kg mouse 24 hrs. p.inf.orally	50	45	10.2 days
None	50	50	8.5 days

Table 20: The effect of the combined application of Tilorone HCl and Poly I:C on experimental TBE in mice.

Group of mice	Treatment	Number of mice inoculated	Number of fatalities	Average survival time
	Experiment Nr. 1 (7 LD_{50})			
Group 1	Tilorone 50 mg/kg 24.hors.pre inf.	50	42	10.1 days
Group 2	Poly I:C 50 mg/kg 3 hrs.p.inf.	50	27	11.5 days
Group 3	Tilorone pre inf. Poly I:C p.inf.	50	26	11.3 days
Group 4	None	50	40	10.4 days

Table 20 (continued):

Group of mice	Treatment	Number of mice inoculated	Number of fatalities	Average survival time
Experiment Nr. 2 (3 LD_{50})				
Group 1	Tilorone 100 mg/kg 24 hrs. pre inf.	50	11	9.5 days
Group 2	Poly I:C 50 mg/kg 3 hrs.pre inf.	50	28	10.7 days
Group 3	Tilorone pre inf. Poly I:C p.inf.	50	11	10.6 days
Group 4	None	50	30	9.9 days
Experiment Nr. 3 (100 LD_{50})				
Group 1	Tilorone 100 mg/kg 24 hrs.pre inf.	50	48	9.2 days
Group 2	Poly I:C 100 mg/kg 3 hrs.p.inf.	50	49	9.2 days
Group 3	Tilorone pre inf. Poly I:C p.inf.	50	46	9.6 days
Group 4	None	50	50	8.9 days

<u>Table 21:</u> Cases of TBE diagnosed between 1964 and 1970 in the Institute of Hygiene, University of Vienna.

Year	Vienna	Lower Austria	Upper Austria	Burgenland	Styria	Carinthia	Total
1964	30	56	9	6	0	0	101
1965	43	67	8	11	0	0	129
1966	34	63	14	15	4	5	136
1967	8	38	7	4	1	2	60
1968	22	30	23	3	0	0	78
1969	26	36	12	10	0	51	135
1970	45	56	27	7	73	112	320

Table 22: Results of the 2-ME test with frozen sera from 1970.

Established recent cases		Suspected recent cases		TBE years ago	
positive*	negative	positive	negative	positive	negative
58 (31.8 %)	124 (68.2 %)	12 (46.2 %)	14 (53.8 %)	0	42 (100 %)

* Decrease at least 2 steps in the HI test after 2-ME treatment.

Table 23: Influence of different concentrations of 2-ME on the titers in the HI test.

Concentration of 2-ME	Serum number and titer			
	1500/70 +	1566/70 +	116/67 ++	117/67 ++
0.00 M	640	160	80	160
0.04 M	160	20	80	160
0.05 M	160	20	80	160
0.06 M	160	20	80	160
0.07 M	160	40	80	80
0.08 M	160	20	80	80
0.09 M	160	20	80	160

+ Sera of patients with recent TBE

++ Sera of patients with TBE years ago

Table 24: Data from 5 fatal cases of encephalitis.

Pat.	Diagnosis	Diagnosis confirmed by	died within
Tol.	TBE	Virus isolation	fow days
Mu	TBE	Serology (CFT)	3-4 wooks
Mal	TBE	Serology (2-ME test)	4 days

Table 25: Interferon in the CNS of fatal cases of encephalitis.

Part of CNS	Patients: Tol.	Mu	Mal.	Control - no encephalitis
Cortex	320[1]	0[2]	160	0
Cerebellum	160	0	n.m.a.	0
Ganglia	n.m.a.	n.m.a.	0	0
Brain stem	160	0	0	0
Cervical cord	320	n.m.a.	0	n.m.a.
Lumbar cord	320	n.m.a.	80	n.m.a.

[1] Reciprocal value of interferon titer

[2] 0 = <80

n.m.a.= no material available

Table 26: Mosquitoes collected at Sandeck (Eastern Neusiedlersee area) in spring 1971 by using sentinel hens.

Date (Period from/to)	Species (number of specimens/number of pools)
March 21 - April 2	Anopheles modestus (1) / 1
April 5 - 11	Culex modestus (11) / 4 Anopheles maculipennis (6) / 3
April 12 - 18	Culex modestus (3) / 1 Anopheles maculipennis (1) / 1
April 19 - 25	Anopheles maculipennis (6) / 2
April 26 - May 2	Anopheles maculipennis (1) / 1
May 3 - 9	- - -
May 10 - 16	Anopheles maculipennis (37) / 2
May 17 - 23	Anopheles maculipennis (43) / 2
May 24 - 30	Anopheles maculipennis (5) / 1

Table 27: Neutralizing antibodies against Tahyna virus in cattle.

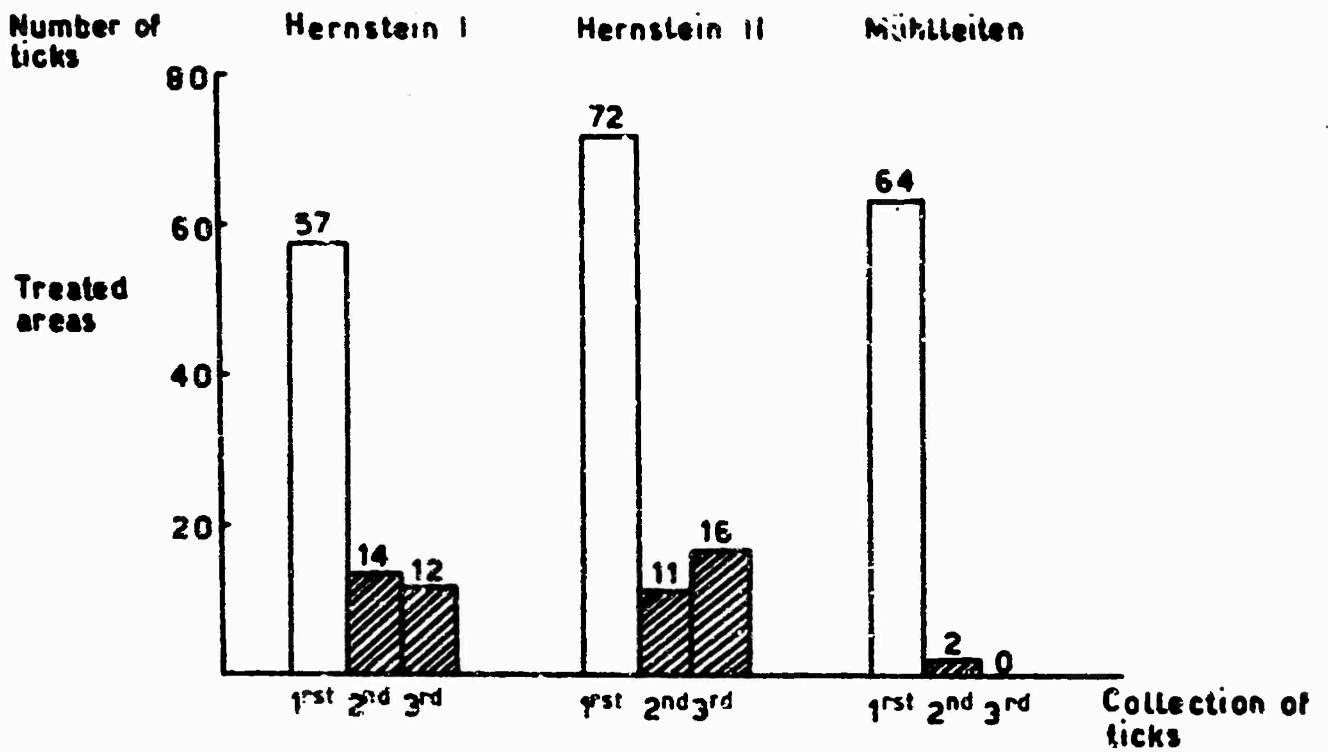
Origin of cattle (community)	Number of sera tested/ number of positive sera	Antibody - rate in %
Frauenkirchen	77 / 16	20.7
Tadten	123 / 5	4
Wallern	110 / 11	10
Pamhagen	143 / 23	16
Illmitz	188 / 20	10.6
T o t a l	641 / 75	11.7

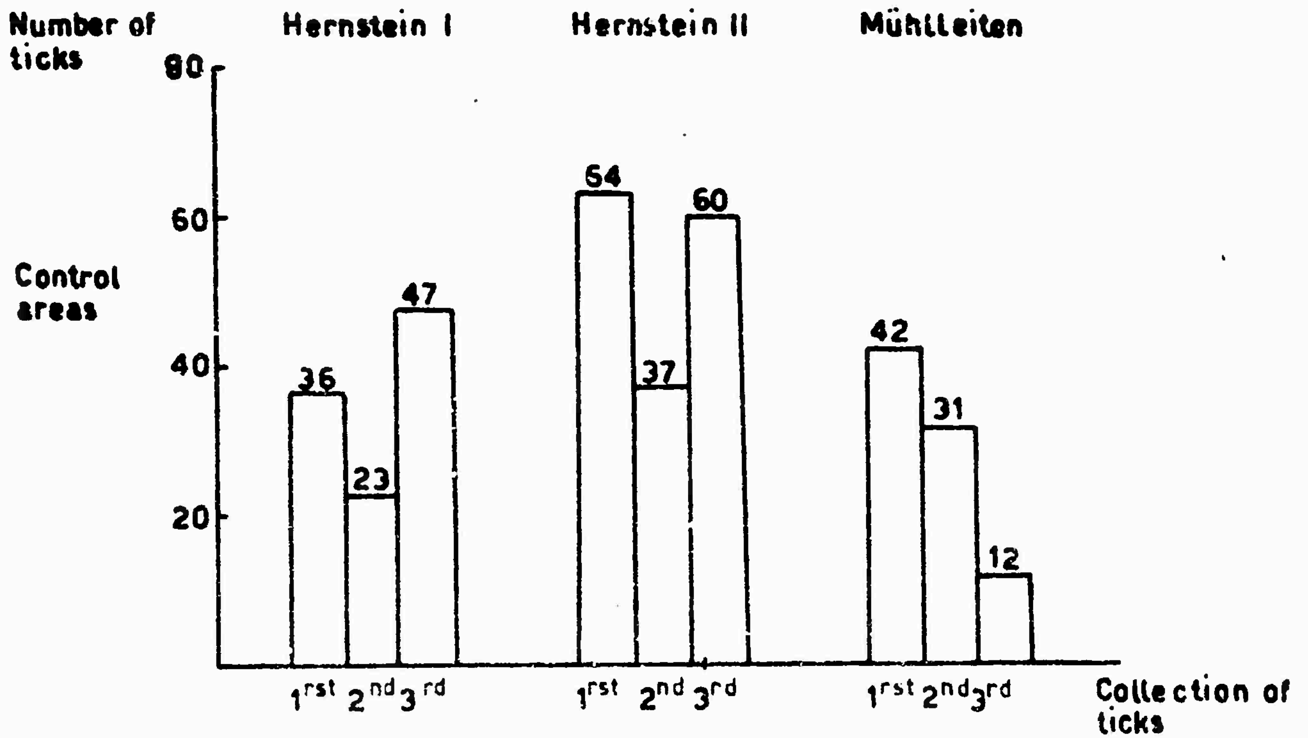
Table 28: Neutralizing antibodies against Calovo virus in cattle.

Origin of cattle (community)	Number of sera tested/ number of positive sera	Antibody - rate in %
Frauenkirchen	77 / 44	57.1
Tadten	123 / 94	76.4
Wallern	110 / 66	60
Pamhagen	143 / 86	60.1
Illmitz	188 / 148	78.7
T o t a l	641 / 438	68.3

Fig.1

Effectiveness of Gardona® against ticks





Number of ticks found in nontreated areas

Number of ticks found after treatment

Fig.2: Content of Semliki Forest virus in the brain and spleen of mice

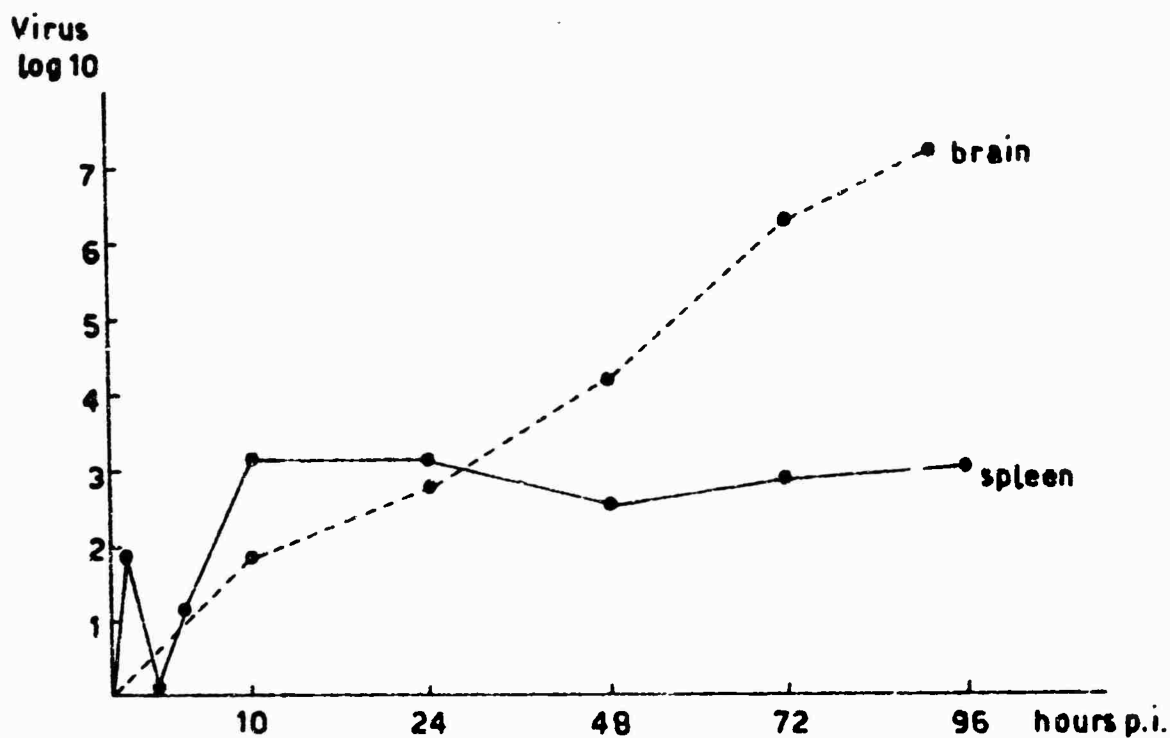


Fig.3: 2,7-bis [2-(diethylamino) ethoxy] fluoren-9-one (Tilorone HCl)

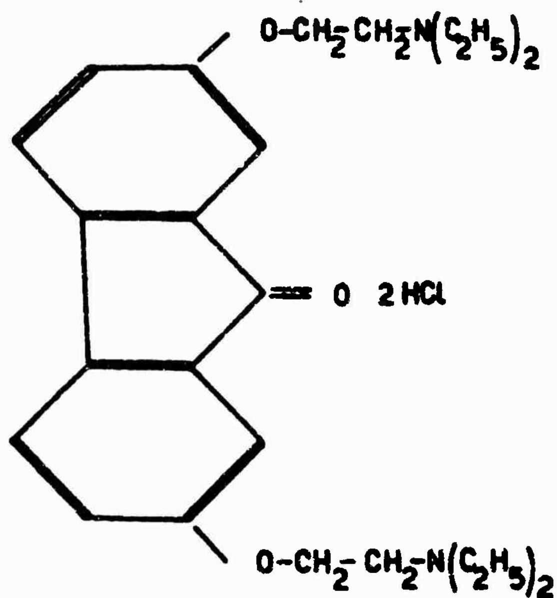
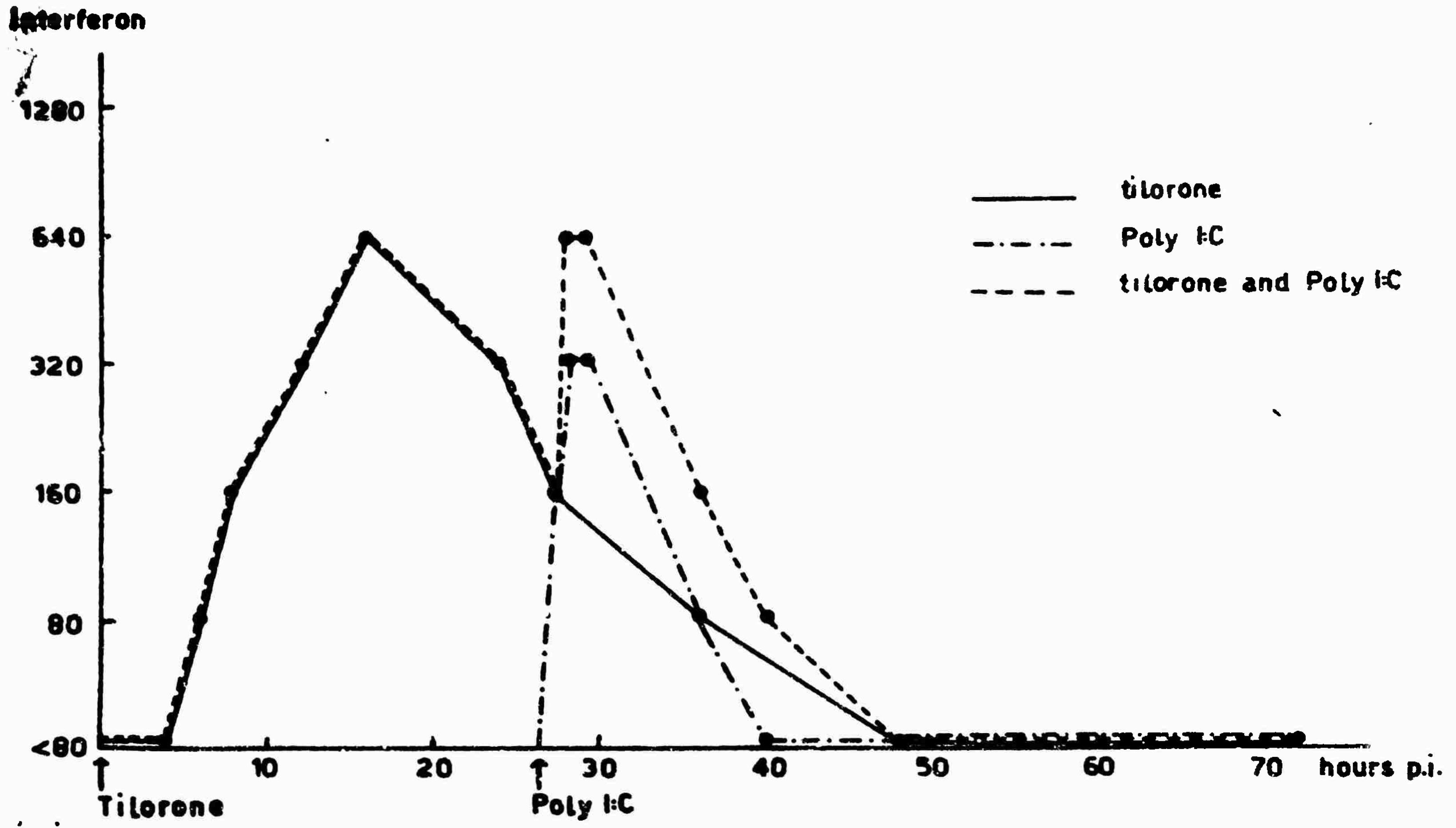
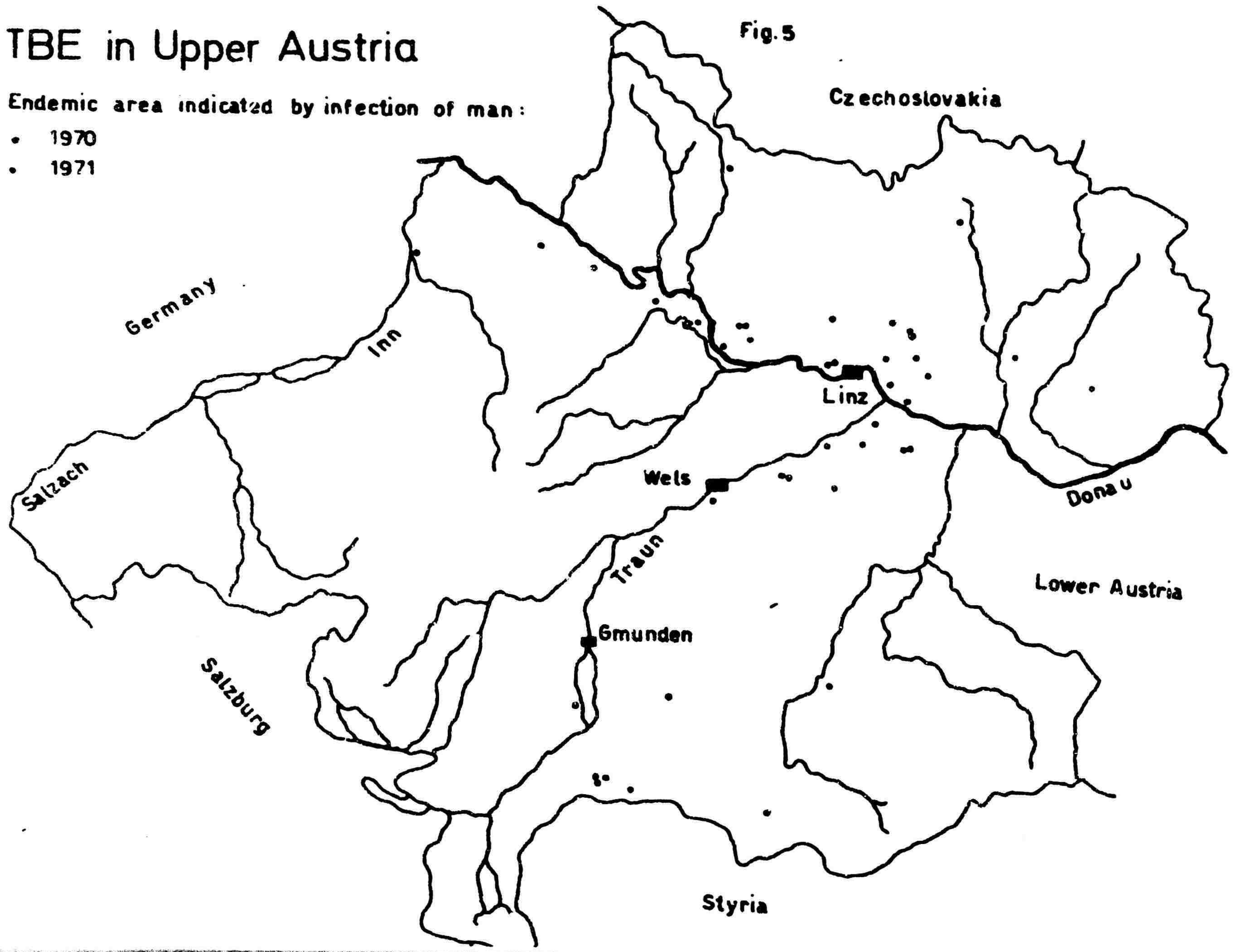


Fig.4: Levels of interferon in the serum of mice after treatment with tilorone (100 mg/kg mouse) and Poly I:C (10 mg/kg mouse)




TBE in Upper Austria
Endemic area indicated by infection of man:
1970
1971
Fig. 5
Czechoslovakia
Germany
Inn
Linz
Wels
Donau
Salzach
Traun
Gmunden
Lower Austria
Salzburg
Styria

TBE in Lower Austria and Vienna

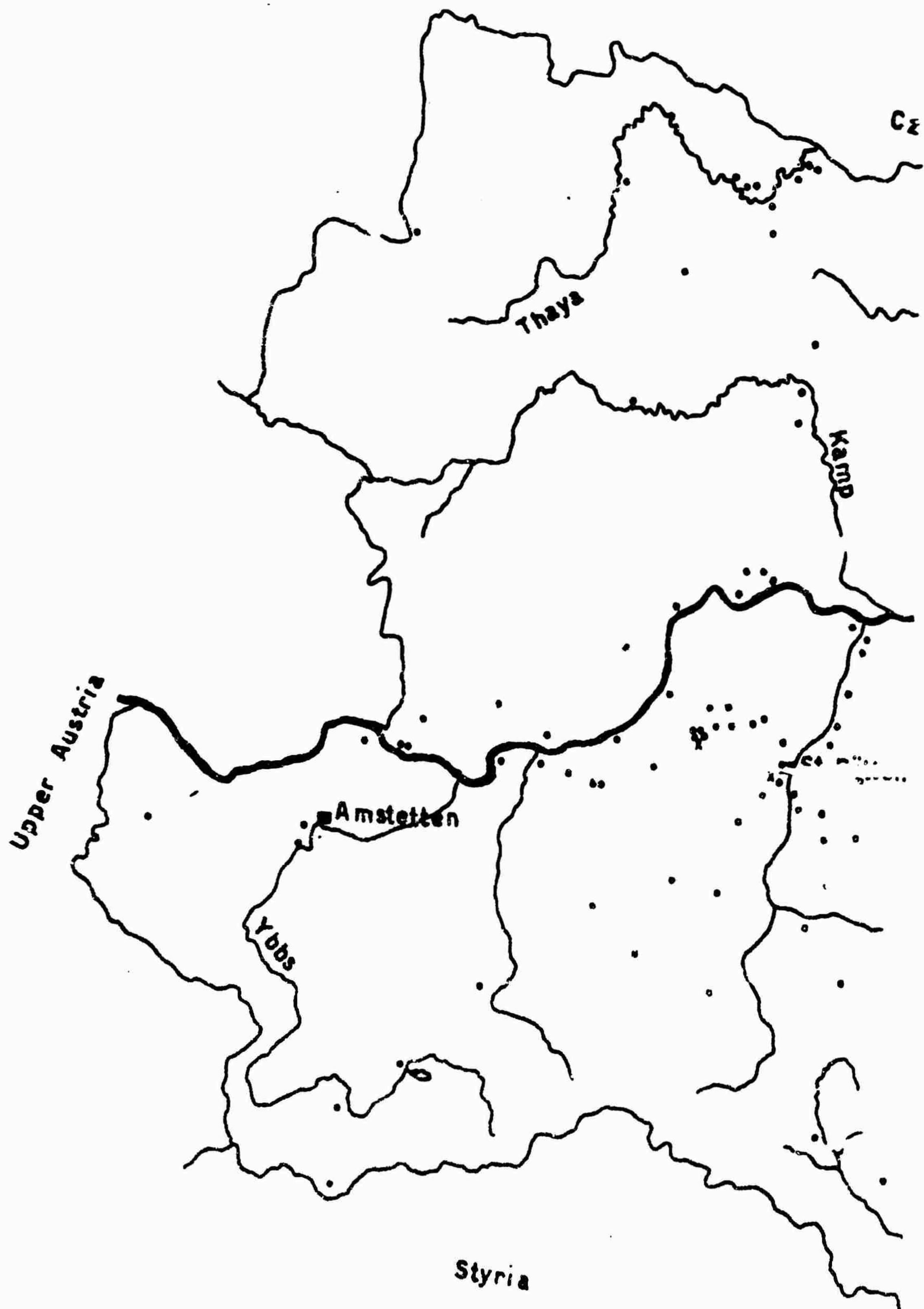


Fig. 6

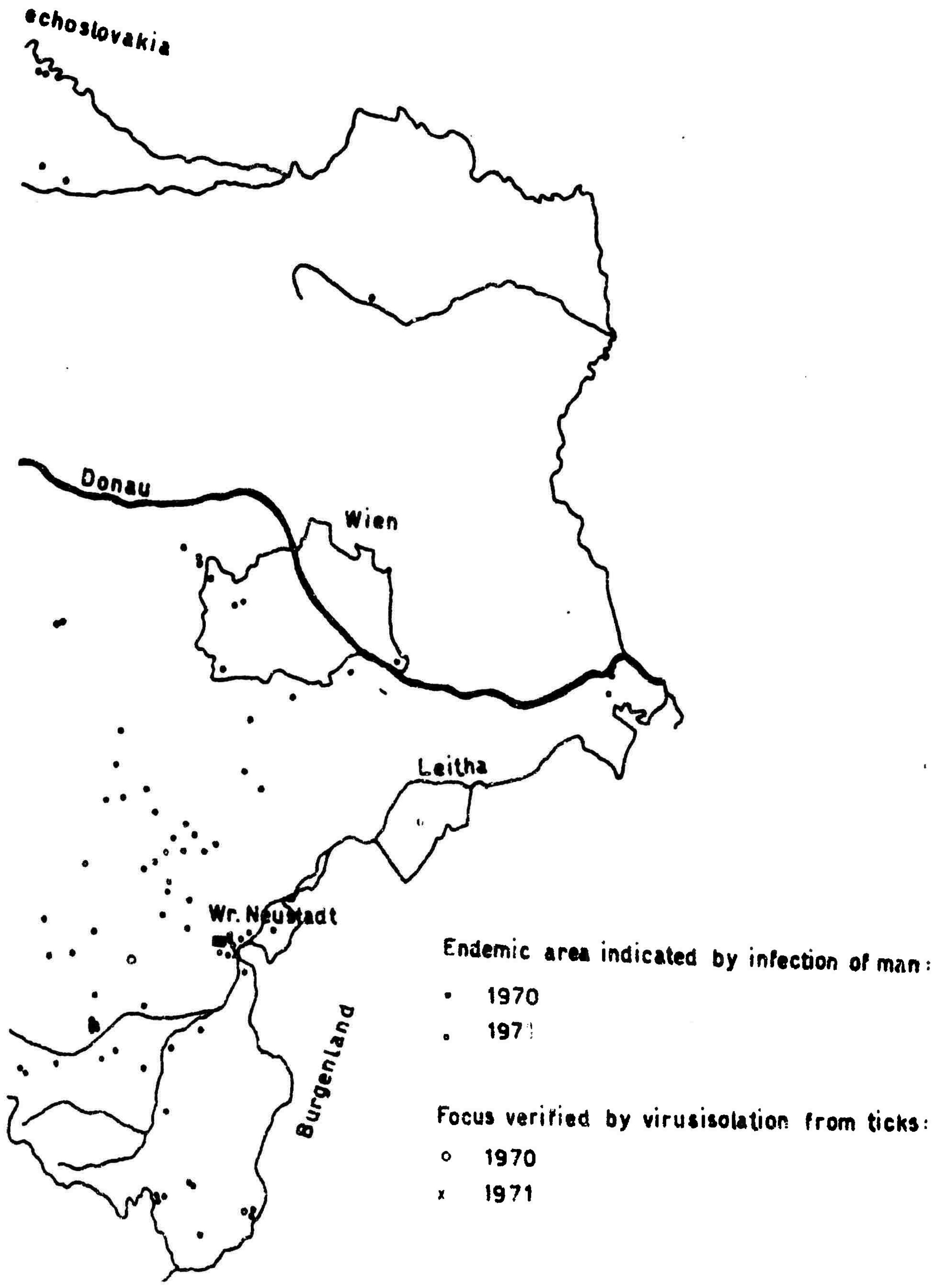


Fig.7

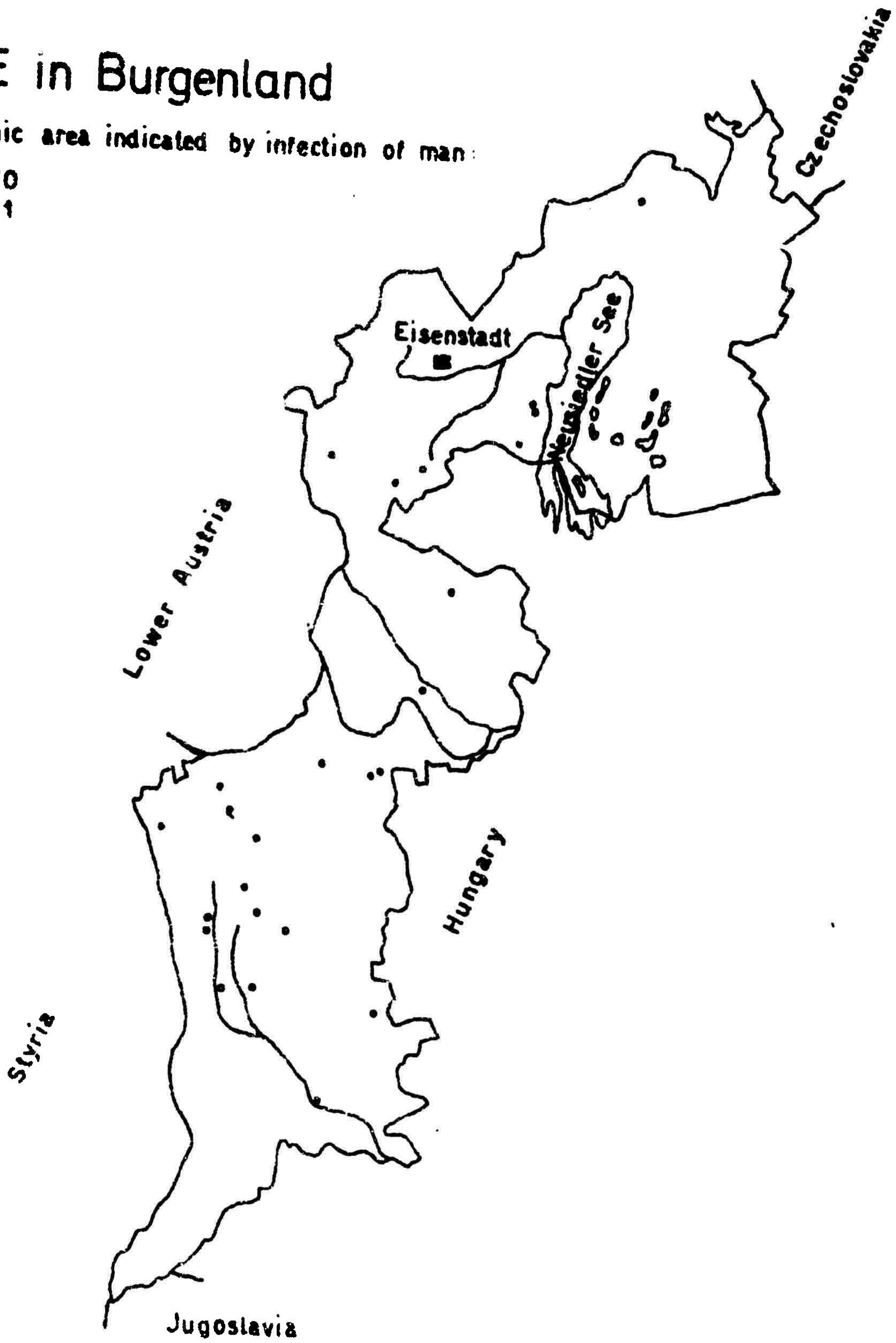


Fig. 8

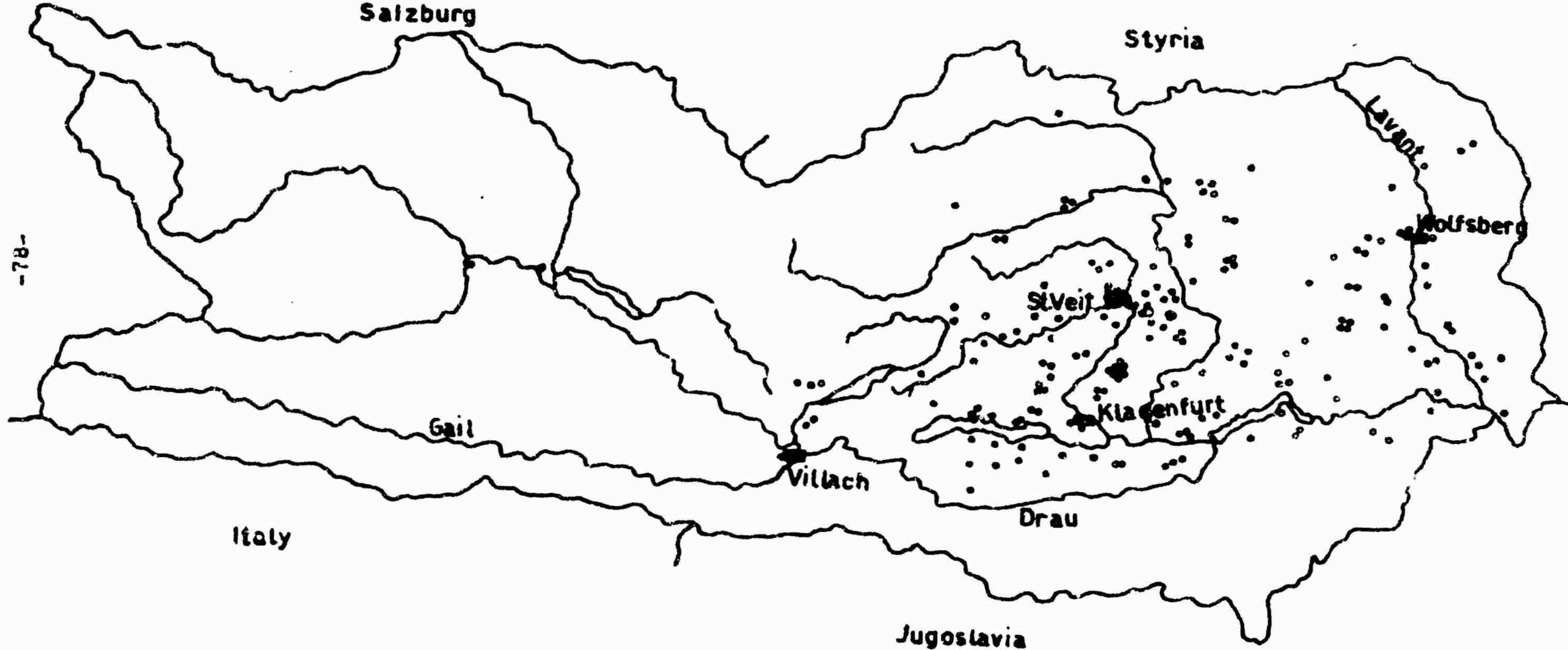
TBE in Carinthia

Endemic area indicated by infection of man:

- • 1970
- • 1971

Focus verified by virusisolation from ticks:

- o 1970



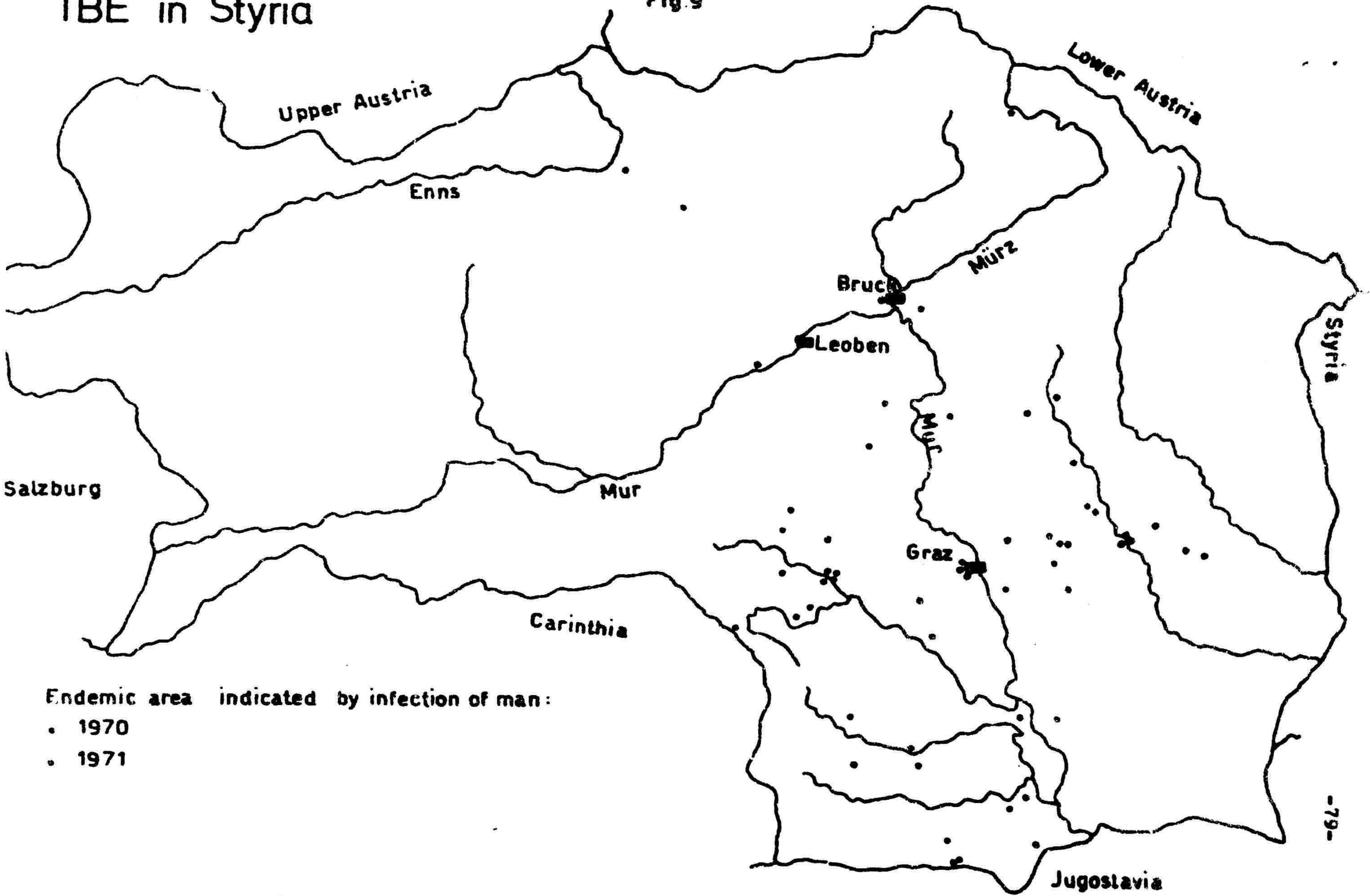

TBE in Styria
Fig.9
Upper Austria
Lower Austria
Styria
Enns
Mürz
Bruck
Leoben
Mur
Mur
Graz
Salzburg
Carinthia
Jugoslavia
Endemic area indicated by infection of man:
• 1970
• 1971

Fig.10: Seasonal fluctuation of mosquitoes collected in cowbarns in the Eastern Neusiedlersee area in 1968 and relative abundance of the most frequent species.
(T resp. C = Isolation of Tahyna resp. of Calovo virus).

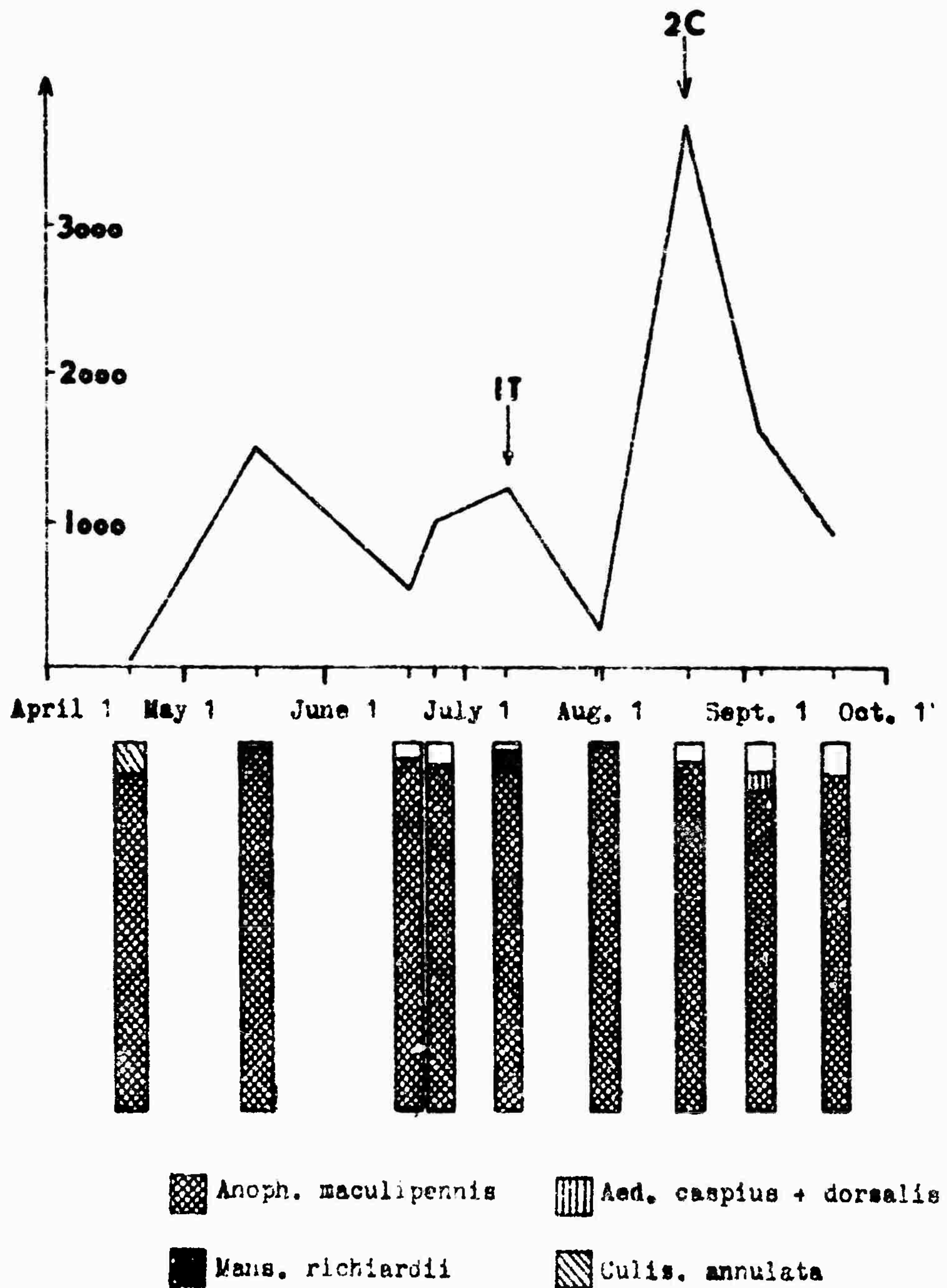
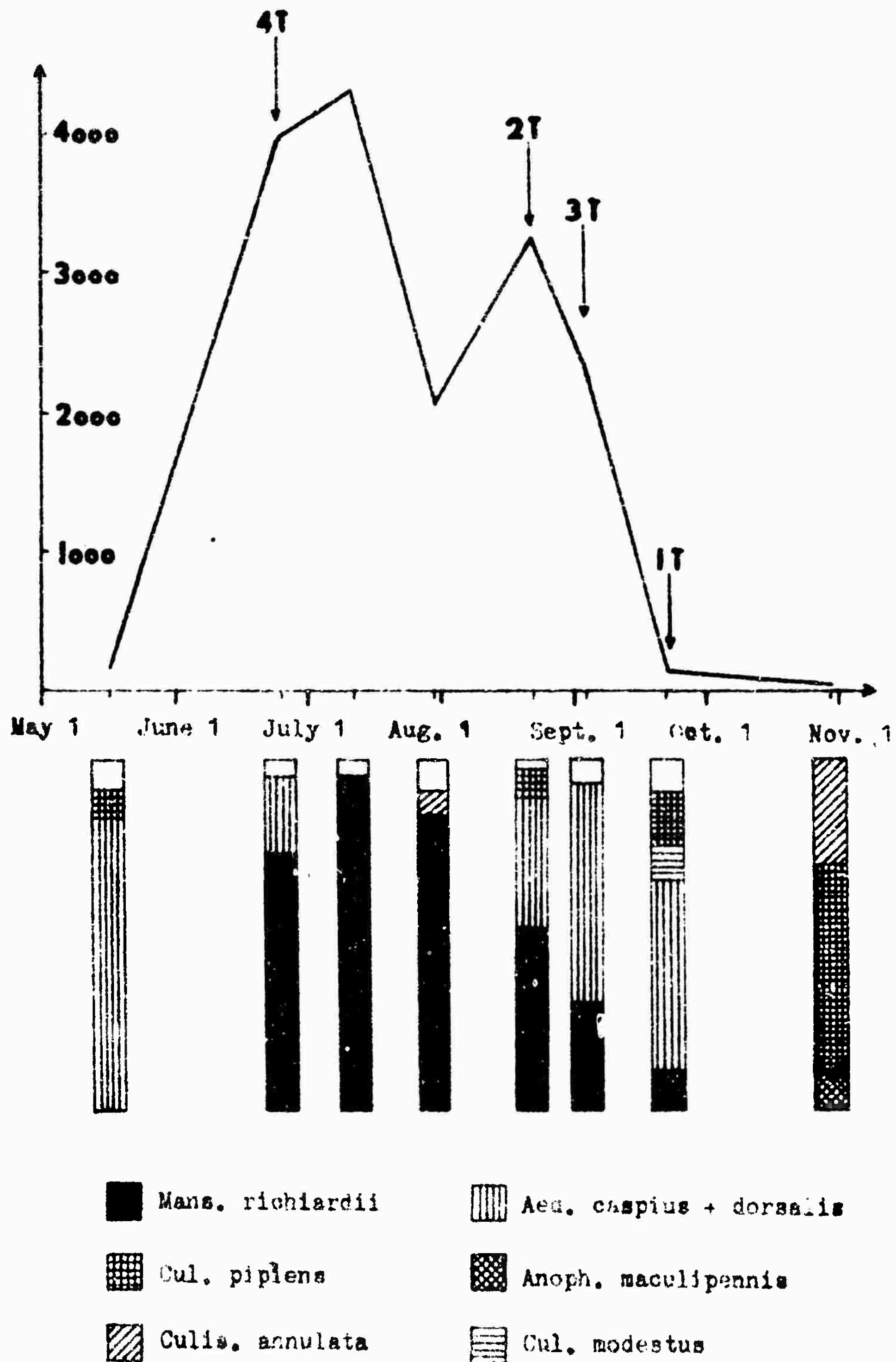


Fig.11: Seasonal fluctuation of mosquitoes collected outdoors in the Eastern Neusiedlersee area in 1968 and relative abundance of the most frequent species
(T = Isolation of Tahyna virus).



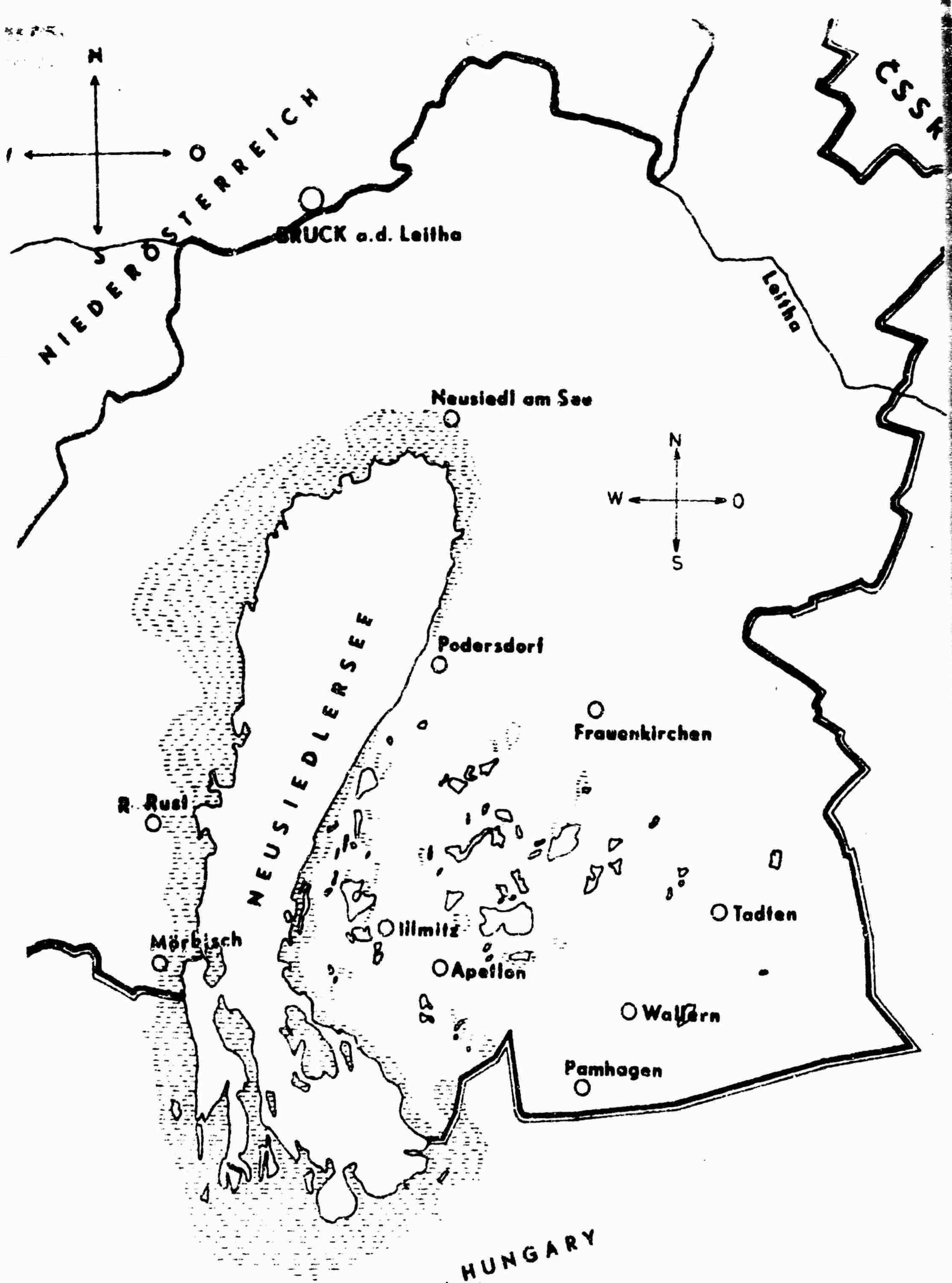
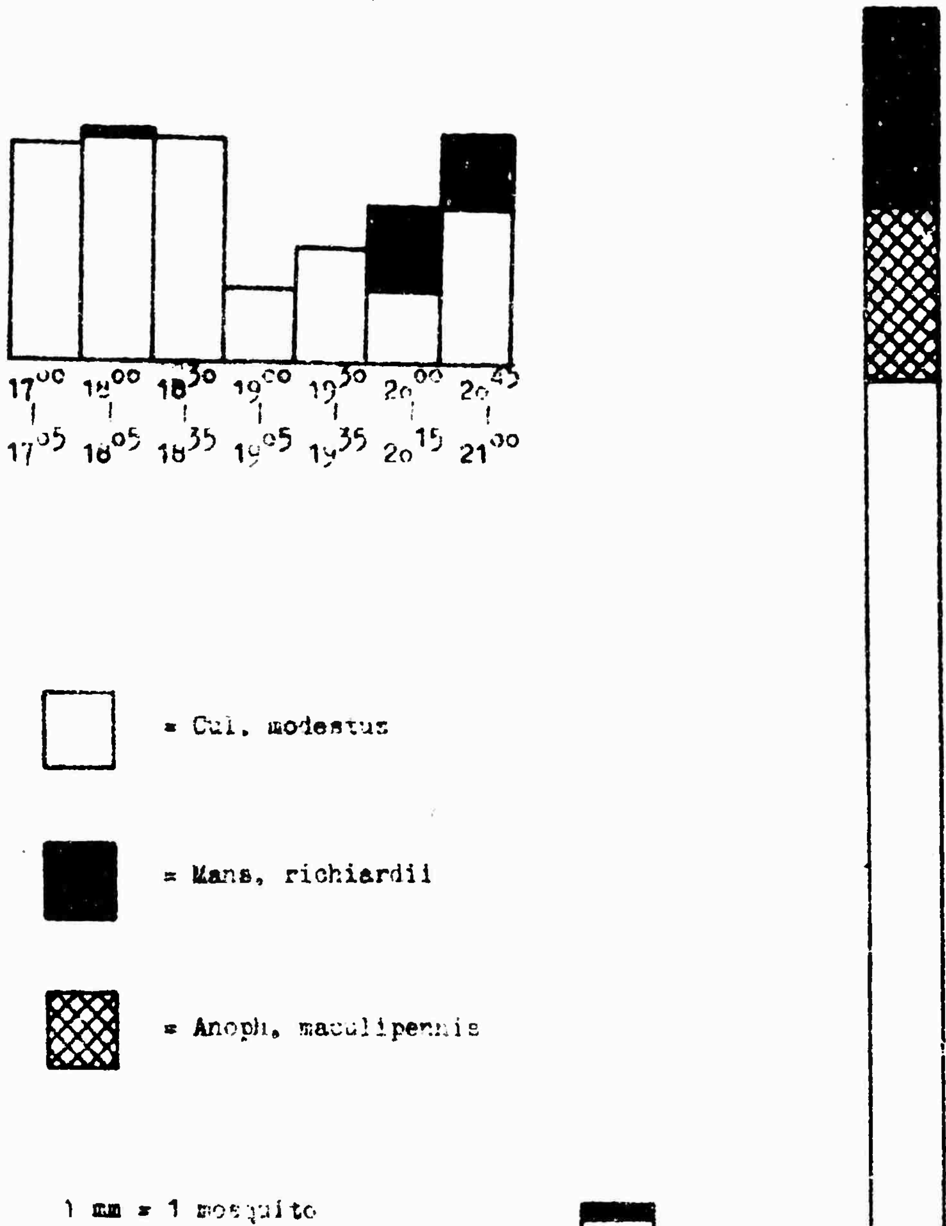


Fig.17: Map of the Neusiedlersee area.

Fig.13: Fluctuation of activity of mosquitoes attacking man near Mörbisch (Western Neusiedlersee area) on August 8, from 5 until 9 p.m.



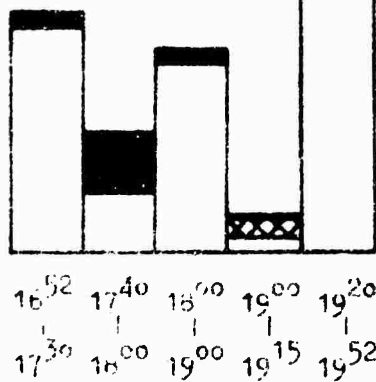


Fig.14: Fluctuation of activity of mosquitoes attacking man near Mörbisch on August 14, from 4.52 until 7.52 p.m.

Fig.15: Fluctuation of activity of mosquitoes attacking man near Mörbisch on August 14 (other point), from 5 until 7.40 p.m.

Fig.16: Fluctuation of activity of mosquitoes attacking man near Mörbisch on August 18, from 7 until 8.30 p.m.

Fig.17: Fluctuation of mosquitoes attacking man near Rust (Western Neusiedlersee area) on August 19, from 7 until 8.15 p.m.

Fig.15

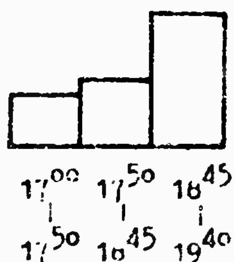


Fig.16

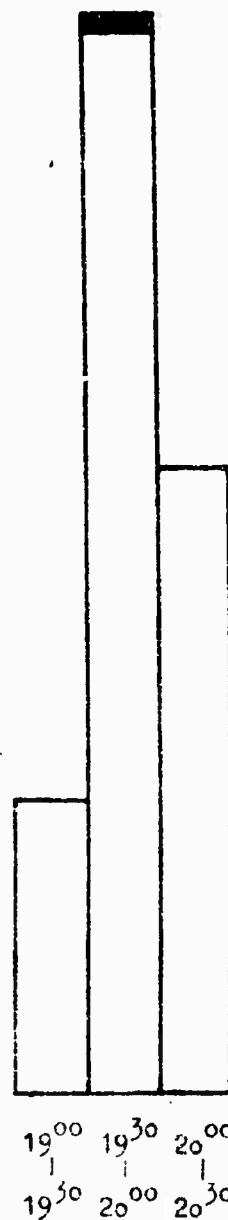


Fig.17

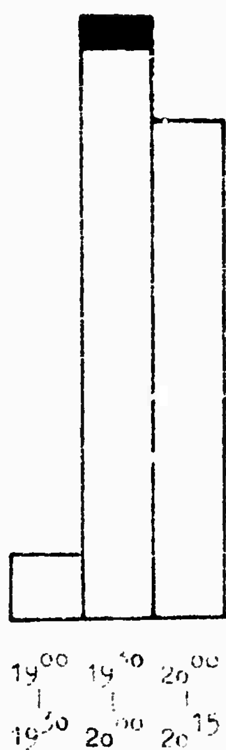
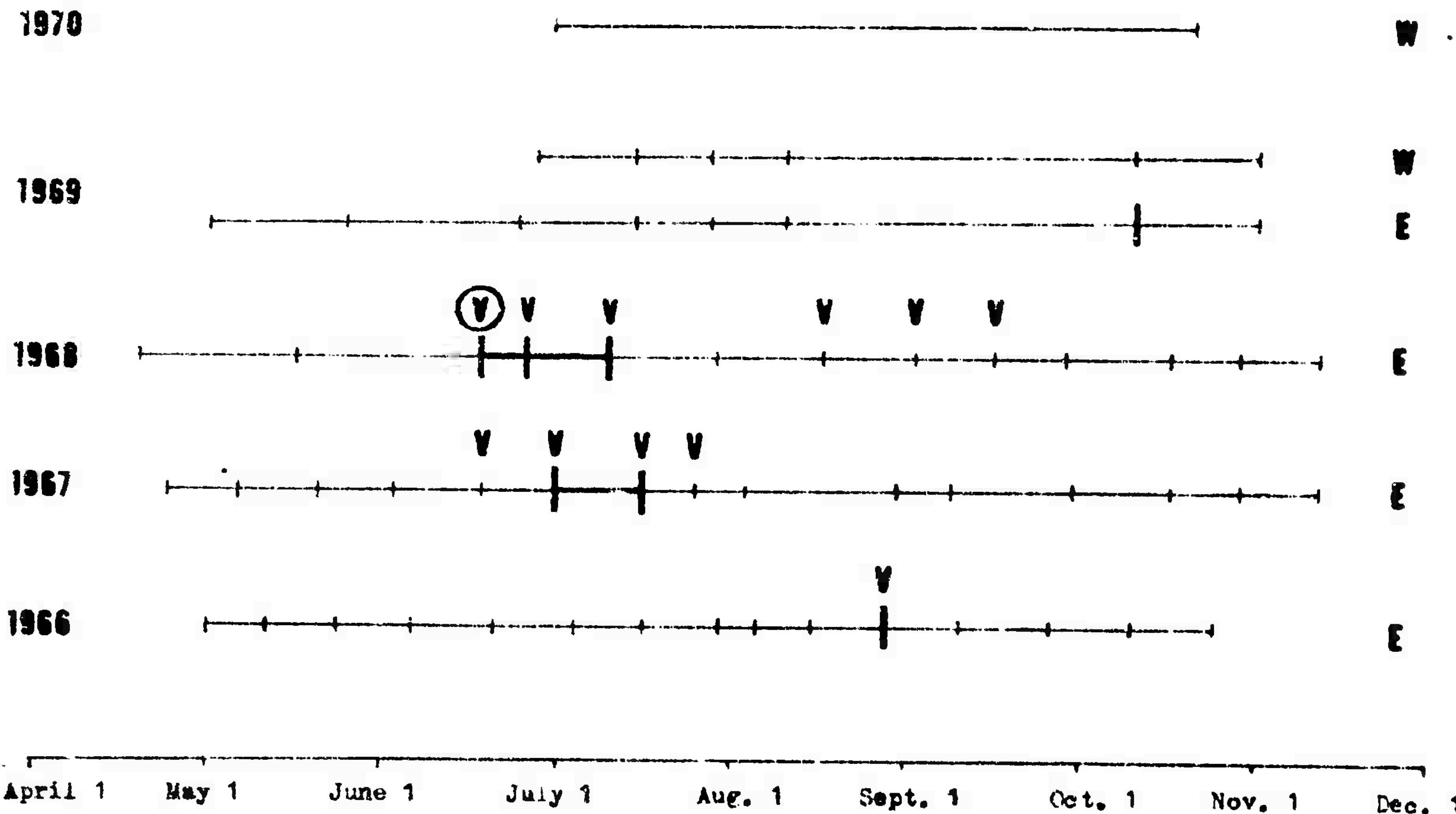


Fig.18: Periods of evidence of Tahyne virus in the years 1966 until 1970. The thin horizontal line shows the duration of exposition of indicator rabbits, the vertical lines indicate dates of bleedings. The thick vertical lines indicate dates of



conversions of antibodies; the thick horizontal lines mark those periods during which neutralizing antibodies were developed. V and Ⓥ indicate virus isolations from mosquitoes respectively from rabbits. E and W=rabbits were exposed in the Eastern resp. in the Western Neusiedlersee

Fig.19: Periods of evidence of Calovo virus in the years 1966 until 1970.
For explanation see Fig.9.