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VOLUME 7 JANUARY, 1963 NUMBER 1

ACTA VIROLOGICA

International Journal

ENGLISH EDITION

CZECHOSLOVAK ACADEMY OF SCIENCES

Declassified in Part - Sanitized Copy Approved for Release 2013/02/13 : CIA-RDP80T00246A021500200001-0

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Acta virol. **7** : 1—6, 1963

Autoradiographic Study of the Penetration of Sendai Virus into the Cell

III. Use of Virus Preparations Labelled with Uracil-C^{14}, P^{32}, Methionine-S^{35} or Cysteine-S^{35}

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Received July 16, 1962

In a previous communication the dynamics of the displacement in the cell of radioactive components of P^{32}-labelled Sendai virus were described (Bukrinskaya *et al.*, 1961). In the present paper this process is characterized quantitatively and the results of experiments with P^{32}-labelled virus are compared with those obtained with Sendai virus labelled with a specific precursor of nucleic acids, namely uracil-C^{14}. To study the fate of viral protein, virus preparations labelled with methionine-S^{35} or cysteine-S^{35} were used.

Materials and Methods

The materials and methods used and the characteristics of the labelled virus preparations were the same as those previously described (Bukrinskaya *et al.*, 1961; Zhdanov and Bukrinskaya, 1961). The labelling of virus with uracil-C^{14} was done by injecting 100 μC of uracil-C^{14} into each chick embryo simultaneously with virus; the virus was purified by a procedure similar to that used previously. Preparations containing $10^{5.0}$—$10^{7.0}$ ID_{50}/ml. of virus were used throughout, thus ensuring a high multiplicity of infection in view of the few thousand cells on the glass strips used in the experiments.

Results

When virus preparations labelled with P^{32} or uracil-C^{14} were used for infection, 0.5—10% of the cells in the culture showed radioactive inclusions. While in autoradiograms prepared 10 minutes after infection of the cells with P^{32}-labelled virus radioactive grains were located predominantly in the nucleolus (Fig. 1), during further incubation the proportion of such cells decreased and cells containing intranuclear and internucleolar grains appeared (Figs. 2 and 3). Two hours after infection the proportion of cells showing grains in the cytoplasm increased (Table 1), the grains being located in the form of conglomerates in the perinuclear part of the cytoplasm (Fig. 4).

To estimate the total radioactivity in the three groups of cells containing cytoplasmic, nuclear or nucleolar grains, the mean number of radioactive grains per cell was determined in an infected human amnion cell culture (Table 2). To characterize the dynamics of incorporation of viral components into cellular structures, the mean number of grains per cell, determined at various intervals, was multiplied by the number of cells in the corresponding group. The results obtained are presented in Figs 6 and 7. It can be seen that the higher the interval elapsed after infection (10—180 minutes), the higher the degree of incorporation of viral components into the nucleus. While during

the first hour after infection the radioactivity of the cytoplasm did not change, two hours after infection it increased rapidly. This increase coincided with the decrease of the number of cells containing grains in the nucleolus. The total radioactivity of the nucleoli however, did not change during incubation,

Table 1. Distribution of radioactive grains in cells infected with P^{32}-labelled Sendai virus

Time after infection in mins	Per cents of cells containing grains in the		
	cytoplasm	nucleus	nucleolus
10	10	10	80
30	11	14	75
60	4	16	80
120	31	18	51
180	32	24	44

Table 2. Mean number of radioactive grains per human amnion cell after infection with P^{32}-labelled Sendai virus

Time after infection in mins	Number of grains in the		
	cytoplasm	nucleus	nucleolus
10	3.2 ± 0.5	11.0 ± 1.5	5.3 ± 0.8
30	5.8 ± 0.7	13.0 ± 2.0	5.2 ± 0.4
60	8.8 ± 1.8	13.6 ± 2.0	6.6 ± 0.8
120	13.2 ± 1.2	16.1 ± 1.3	5.0 ± 0.3
180	11.1 ± 1.5	16.1 ± 1.8	10.0 ± 1.9

this fact being caused by a parallel increase of the number of intranucleolar grains during prolonged incubation of infected cells (Fig. 7).

When cells were treated for 10—60 minutes with noninfected allantoic fluid labelled to the same degree as the virus preparations used for infection, grains were not detected in the cells. After 2—3 hours' contact some of the cells contained grains in the nucleus. P^{32} was not incorporated into the nucleoli of such control cells.

The character of the incorporation of the virus into the structural components of the cell was the same, regardless whether cultures of stable cell lines (Cynomolgus monkey heart or human amnion cells) or primary cultures of chick embryo cells were used.

Experiments with uracil-C^{14} were carried out in human amnion cell cultures. The character of the incorporation of viral components into cellular structures was the same as with virus preparations labelled with P^{32} (Table 3), but the proportion of cells containing grains in the nucleolus was lower duirng the early period of incubation.

When human amnion cells were treated for 2 hours with noninfected allantoic fluid labelled with uracil-C^{14} and diluted to the same degree as the virus preparations used for infection, some of the cells contained grains in the nucleus. After 3 hours an appreciable proportion of cells contained radioactive grains,

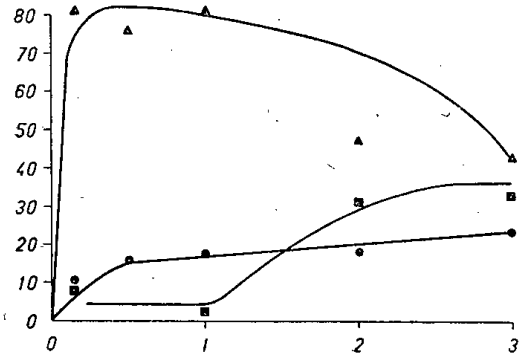

Fig. 6.

Proportion of cells (%) in culture infected with P^{32}-labelled Sendai virus showing incorporation of radioactive viral components in relation to the incubation time ▪ cytoplasm, ● nuclei, ▲ nucleoli; ordinate: % of cells; abscissa: hours of incubation.

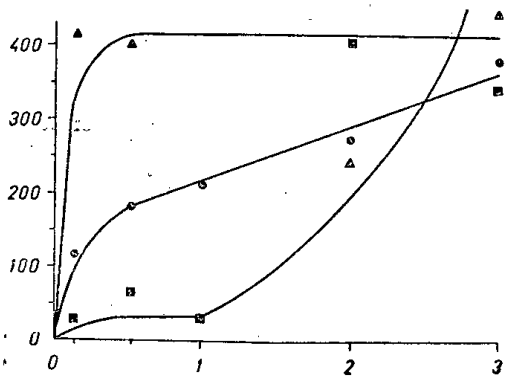

Fig. 7.

The change of the total radioactivity of cells (proportion of cells in % showing radioactive grains × mean number of grains per cell) infected with P^{32}-labelled Sendai virus during incubation ordinate: cells × grains; for other explanations see Fig. 5.

Table 3. Distribution of radioactive grains in cells infected with uracil-C^{14}-labelled Sendai virus

Time after infection in mins	Per cents of cells with grains in the		
	cytoplasm	nucleus	nucleolus
10	6	34	60
30	11	38	51
60	14	45	41
120	18	50	32

90% of them showing intranuclear and only 10% intranucleolar localisation of the grains.

Using virus preparations labelled with methionine-S^{35} or cysteine-S^{35}, faint grains distributed uniformly over the nucleus and cytoplasm appeared on the surface of most of the cells already 10 minutes after infection (Fig. 5). The number of grains reached a maximum 60 minutes after infection. The uiform distribution of grains suggest that they represented viral components adsorbed onto, but which had not penetrated into the cell. To verify this assumption, cells were inoculated in parallel experiments with methionine-S^{35}-labelled virus at 4 and 37° C, taking into account that the penetration of virus into the cells is markedly suppressed at low temperatures. The number of grains

per cell was 21.0 ± 2.5 and 24.0 ± 3.0 at 37 and 4° C, respectively, i.e. about the same at both temperatures. Similar results were obtained when virus preparations labelled with cysteine-S^{35} were used.

When cultures were inoculated at 4° C with uracil-C^{14}-labelled virus, the proportion of cells showing incorporation of viral components as well as the occurrence of intranucleolar grains was decreased (Table 4).

Table 4. The effect of temperature on the incorporation of Components of uracil-C^{14}-labelled Sendai virus into human amnion cells

Temperature °C	Proportion of cells containing grains	Percentage of cells with grains in the		
		cytoplasm	nucleus	nucleolus
37	10%	5	44	51
4	2%	36 (over the whole cell)	44	20

Discussion

Analysis of virus preparations labelled with P^{32} or S^{35} has shown that the distribution of the radioactivity between the viral components was not uniform. While 44—58% of the activity was found in the S-antigen (internal ribonucleoprotein) fraction of P^{32} labelled virus, only 16% was recovered in the haemagglutinin and denatured protein fractions obtained after splitting of the virus with ether. In virus preparation labelled with S^{35}, 55.5% of the radioactivity was bound to the viral protein and only 10.4% was found in the S-antigen (Bukrinskaya *et al.*, 1961). Thus the grains contained in cells infected with P^{32}- or S^{35}-labelled virus corresponded essentially to the nucleic acid and protein fraction of the virus, respectively. Labelling with uracil-C^{14}, a specific precursor of nucleic acids, enabled a more reliable examination of the fate of viral nucleic acid.

While 97% of the cells in the culture inoculated with methionine-S^{35} and cysteine-S^{35}-labelled virus showed grains on autoradiograms, only 0.5—10% of the cells contained radioactive inclusions after inoculation with P^{32}- or uracil-C^{14}-labelled virus. In the former case the grains were uniformly distributed over the whole cell, while in the latter a tendency for accumulation of the grains in the nucleolus was evident. These findings can be readily explained when it is assumed that the nucleic acid component of the virus penetrated into the cell, while the viral protein remained on its surface. The results obtained in experiments on the adsorption of S^{35}-amino acid or uracil-C^{14}-labelled viruses at different temperatures supported this assumption. But the data obtained do not exclude that a part of the virus protein penetrates into some cells. This question could be solved by autoradiography of sectioned cells.

Taking into account the fact that one hours' adsorption was sufficient to obtain optimum virus multiplication in the cells, it can be deduced that the

changes observed in the localization of grains during incubation of the infected cultures were due to displacement of viral components already penetrated into the cell rather than to the penetration of new virus particles, although the latter factor cannot be completely excluded. The results of the present experiments indicate that a part of the viral components which had penetrated into the nucleolus were liberated during further incubation and concentrated in the nucleus. Then they entered the cytoplasm and were located in the perinuclear zone.

Recently autoradiographic studies on the synthesis of cellular ribonucleic acids have been published (Errera, 1961; Perry *et al.*, 1961). Their results are in agreement with the data given in the present paper. Thus the fate of viral ribonucleic acid which had penetrated into the cell is essentially the same as that of cellular ribonucleic acid in a noninfected cell. Some hours after the entry of viral ribonucleic acid from the nucleus into the cytoplasm an intensive synthesis of viral antigen takes place (Zhdanov *et al.*, 1961). In agreement with other investigators (Brachet, 1960; Errera, 1961; Jacob and Monod, 1961) we can asume that viral ribonucleic acid possibly plays the role of messenger ribonucleic acid in the synthesis of specific protein in the cytoplasm.

Summary

1. The dynamics of the displacement of the nucleic acid component of P^{32}- or uracil-C^{14}-labelled Sendai virus in the infected cell was studied by autoradiography. First radioactive grains appeared early after the infection mainly in the nucleolus, then in the nucleus, and 2 hours after infection in the cytoplasm. The proportion of the cells in the culture showing incorporation of viral components by autoradiography was 0.5—10%.

2. In autoradiograms of cells infected with methionine-S^{35} or cysteine-S^{35} labelled Sendai virus the radioactive grains were uniformly distributed on the surface of 90—97% of the cells in the culture.

3. The interaction of methionine-S^{35} or cysteine-S^{35}-labelled virus and cells at 4° C instead of 37° C, did not lead to decreased labelling of the cells, while the radioactivity of the cells was markedly reduced when the cells were inoculated at 4° C with uracil-C^{14}-labelled virus.

4. The results obtained indicate that after inoculation the ribonucleic acid or the internal ribonucleoprotein (S-antigen) of Sendai virus penetrated into the cells, while the protein coat of the virus remained on their surface.

Acknowledgement. The authors thank Dr. A. S. Konikova and her collaborators from the Laboratory of Biochemistry of the Institute of Surgery, U.S.S.R. Acad. Med. Sci., and Prof. V. K. Modestov and his collaborators from the Chair of Medical Radiology of the Central Institute of Postgraduate Training of Physicians for the aid in this study.

References

Brachet, J. (196): Le rôle biologique d'acides nucléiques. *R. C. Ist. Sci. Camerino* **1**, 3.

Bukrinskaya, A. G., Zhdanov, V. M., and Ramenskaya, G. P. (1961): Autoradiographic study of the penetration of Sendai virus into the cell. II. Use of virus preparations labelled in the P^{32}. *Vop. Virusol.* **6**, 547.

Errera, M. (1961): Biochemical processes in injured cells in relation to cell recovery. *J. cell. comp. Physiol.* **58** (Supplem.), 209.

Jacob, F., and Monod, J. (1961): Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**, 318.

Perry, R. P., Hell, A., and Errera, M. (1961): The role of the nucleolus in RNA and proteins synthesis. I. Incorporation of cytidine into normal and nucleolar inactivated HeLa cells. *Biochem. biophys. Acta* **49**, 47.

Zhdanov, V. M., and Bukrinskaya, A. G. (1961): Autoradiographic study of the penetration of Sendai virus into the cell. I. Labelling of Sendai virus with radioactive isotopes. *Vop. Virusol.* **6**, 542.

Zhdanov, V. M., Bukrinskaya, A. G., and Azadova, N. B. (1961): Fluorescent microscopic study of incomplete Sendai virus formation in tissue culture cells. *J. Immunol.* **87**, 641.

Explanation of Photomicrographs:

Fig. 1. Autoradiogram of a human amnion cell infected with P^{32}-labelled Sendai virus.
Time after infection: 10 mins; grains are located on the cytoplasmic and nuclear membranes, and in the nucleolus; × 900.

Fig. 2. Autoradiogram of human amnion cells prepared 1 hour after infection with P^{32}-labelled Sendai virus.
The upper left cell contains grains located in the internucleolar zone of the nucleus; × 600.

Fig. 3. Autoradiogram of human amnion cells prepared 1 hour after infection with P^{32}-labelled Sendai virus.
Isolated nuclei containing radioactive grains; × 600.

Fig. 4. Autoradiogram of human amnion cells infected with P^{32}-labelled Sendai virus. Two hours after infection. The grains in the left cell are located in the perinuclear zone of the cytoplasm. × 600.

Fig. 5. Autoradiogram of human amnion cells prepared 1 hour after infection with methionine-S^{35}-labelled Sendai virus.

Acta virol. **7** : 7—15, 1963

Inactivation of Aujeszky Disease (Pseudorabies) Virus by Nitrous Acid

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Received August 17, 1962

Mundry and Gierer (1958) showed that desamination of purine and pyrimidine bases of isolated viral ribonucleic acid (RNA) by treatment with nitrous acid (HNO_2), carried out as described by Schuster and Schramm (1958), led in addition to inactivation also to induction of mutations. They demonstrated, too, that mutants can be obtained also when intact viruses are treated with HNO_2. Recently the kinetics of inactivation of various RNA- or deoxyribonucleic acid (DNA)-containing viruses by HNO_2, the properties of isolated chemical mutants and those of inactivated virus were studied by several investigators (Boeyé, 1959; Tessman, 1959; Vielmetter and Wieder, 1959; Schäfer *et al.*, 1959; Granoff, 1961; Bautz-Freese and Freese, 1961; Carp and Koprowski, 1962; Restle *et al.*, 1962; Wassermann, 1962). We studied the reaction between HNO_2 and the Aujeszky disease virus, a DNA-containing animal virus (Ben-Porat and Kaplan, 1962). In the present paper the kinetics of inactivation of this virus at different pH, temperatures and HNO_2 concentrations are described. The immunogenicity of the inactivated virus was also investigated.

Materials and Methods

Virus. The Bucharest strain of Aujeszky disease virus in its 368th—372nd chick embryo cell (CEC) culture passage was used throughout. Virus suspensions were prepared as follows. Bottles were seeded with 5×10^7 CEC in 100 ml. of growth medium (1 : 1 mixture of medium 199 and Earle's saline, containing 2% calf serum heated at 56° C for 30 minutes) (PEC) and incubated at 37° C for 48 hours. The medium was then removed and the cells inoculated with about 10^8 plaque forming units (PFU) of virus. The virus was allowed to adsorb at 37° C for 90 minutes. Then 100 ml. of fresh PEC medium were added and the cultures incubated at 37° C for a further 48 hours. The harvested infectious culture fluids, containing about 5×10^7 PFU of virus per ml., were freed of cells and their debris by low speed centrifugation.

In some experiments virus partially purified by sedimentation at 40,000 × g for 30 minutes was used. The pellet was resuspended in phosphate buffered saline pH 7.4 (0.007M phosphate, 0.14M NaCl).

Infectivity titration. Virus was titrated by the plaque method in CEC monolayers as described by Škoda and Mayer (1961) using 2—4 plates for each serial tenfold dilution. After adsorption of the virus at room temperature for 90 minutes the inoculum was removed. In some experiments also the $TCID_{50}$ titres of the virus samples were estimated. Tube cultures containing 5×10^5 CEC and 1 ml. of PEC medium were inoculated with 0.1 ml. of serial tenfold dilutions of the samples using 4 tubes for each dilution. The cultures were incubated at 37° C and the cytopathic effect was read after 5 days.

Neutralisation test. Serial twofold dilutions of rabbit immune serum heated at 56° C for 30 minutes were mixed with equal volumes of a suspension containing 10^3 $TCID_{50}$ of virus per ml. and incubated at 37° C for 60 minutes. Tube cultures of CEC were then inoculated with 0.2 ml. of the mixture using 4 tubes for each dilution. The titres were taken as the reciprocal of the highest initial dilution of serum showing complete inhibition of the viral cytopathic effect.

Treatment of virus by HNO_2. Two parts of virus suspension were mixed with one part of 1M acetate buffer of required pH and one part of a 4M $NaNO_2$ solution in distilled water. When solutions of lower $NaNO_2$ concentration were used, NaCl was added to adjust the concentration

of Na^+ to 4M. All solutions were heated to the required temperature before mixing. The reaction mixture was kept in a water bath of a constant temperature and stirred by a magnetic stirrer. The pH of the mixture did not change during the reaction time. Samples were withdrawn at intervals, diluted 1 : 10 with 0.25M phosphate buffer pH 7.85 to stop the reaction and immediately assayed for infectivity. The final pH of the 1 : 10 diluted virus suspension was about 7.4. $NaNO_2$ was not removed from the samples, because an appreciable drop in virus titre was observed regularly after exhaustive dialysis against phosphate buffered saline pH 7.4. Controls set up in parallel consisted of virus, acetate buffer and 4M NaCl.

Several batches of inactivated virus for immunisation were prepared by treatment of crude virus suspension for 30 minutes with 1M $NaNO_2$ at pH 5.55 and 37° C. The suspension was then dialysed exhaustively against tap water, the viral antigen sedimented at 40,000 × g for 30 minutes, resuspended in 1/10 of the original volume of phosphate buffered saline pH 7.4 and freed of insoluble material by low speed centrifugation. No live virus could be demonstrated in such preparations by the plaque method, when 0.4 ml. of the suspension were plated on CEC monolayers.

Experimental

The character of the inactivation reaction

Most of the curves characterizing the decrease of infectivity with time showed essentially an exponential rate of inactivation (Figs. 1 and 2) corresponding to the equation

$$\log (I_0/I) = k \times t + b \,, \qquad (1)$$

I_0 being the infectivity titre of control virus suspension at zero time, I that of treated virus at time t and k the reaction rate constant (min.$^{-1}$). However

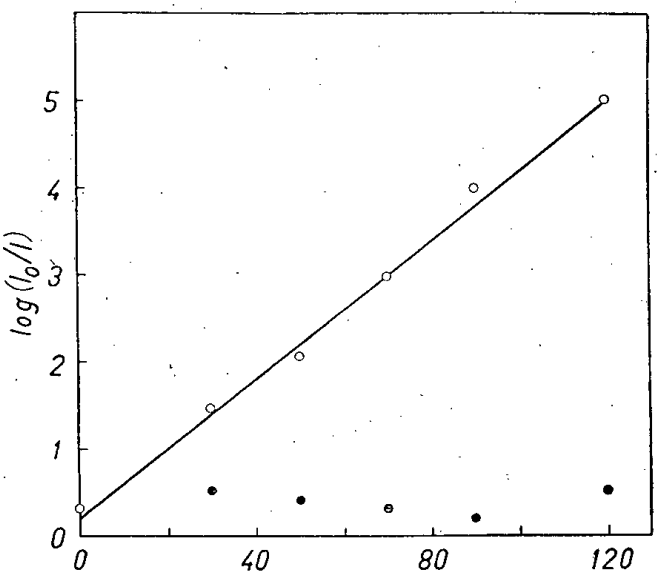

Fig. 1.

Inactivation of Aujeszky disease virus by HNO_2
1M final concentration of $NaNO_2$, pH 5.55, 20° C;
abscissa: time in minutes; ● = controls; infectivity assayed by the plaque method;
$k = 0.0397$ min^{-1}

almost in all experiments the titre of virus suspension determined immediately after mixing with $NaNO_2$ solutions was significantly lower than the titre of control virus suspension at zero time and therefore the constant b was usually

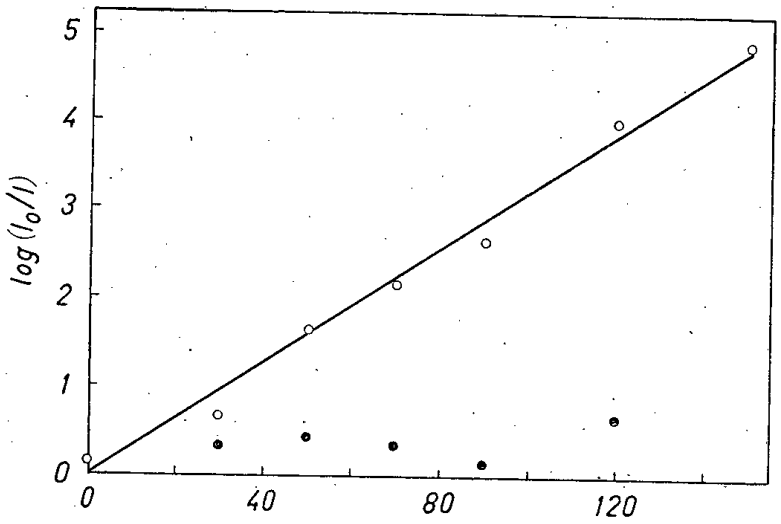

Fig. 2

Inactivation of Aujeszky disease virus by HNO_2
Conditions as in Fig. 1; $TCID_{50}$ estimated in tube cultures; $k = 0.0327\ min^{-1}$

higher than zero (Fig. 3). In some experiments, especially at low temperatures and concentrations of HNO_2, an initial shoulder was observed in the inactivation curve (Fig. 4) followed by exponential inactivation according to equation (1). In such cases the rate constant was calculated from the exponential portion of the curve.

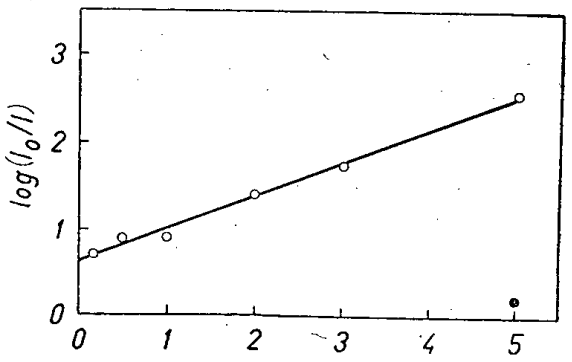

Fig. 3.

Inactivation of Aujeszky disease virus by HNO_2 1M final concentration of $NaNO_2$, pH 5.1, 20° C; for further explanations see Fig. 1; $k = 0.378\ min^{-1}$

Impurities of nonviral origin contained in crude virus preparations did not affect the appearance of the shoulder or the rate of inactivation. At 1M final concentration of $NaNO_2$, pH 5.7 and 20° C k was e.g. 0.0312 and 0.0310 for crude and partially purified virus, respectively. When a pure virus line, obtained by threefold plaque purification, was used instead of wild virus, the shoulder did not disappear.

It was demonstrated that under identical conditions the cytopathic activity

of virus and its ability to form plaques were inactivated at about the same rate (compare Figs. 1 and 2). Therefore in further experiments infectivity was assayed only by the more exact plaque method.

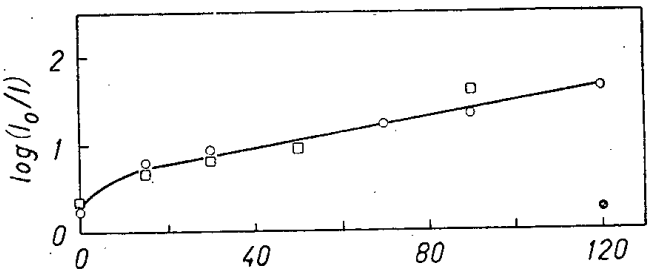

Fig. 4.
Inactivation of Aujeszky disease virus by HNO_2
1M final concentration of $NaNO_2$, pH 5.55, 4° C; for further explanations see Fig. 1; note the initial shoulder in the inactivation curve; $k = 0.00872\ min^{-1}$

The relationship between temperature and the inactivation rate

The dependence of the inactivation rate on temperature was studied at pH 5.55 and a 1M final concentration of $NaNO_2$. When log k was plotted against the reciprocal of absolute temperature (1/T), the experimental points fitted well the Arrhenius equation

$$\log (k/k_1) = \frac{q}{2.303 \times R}\left(\frac{1}{T_1} - \frac{1}{T}\right), \qquad (2)$$

where R is the gas constant and q the activation energy. The latter was 15.2 kcal/mole, as determined from the slope of the line shown in Fig. 5.

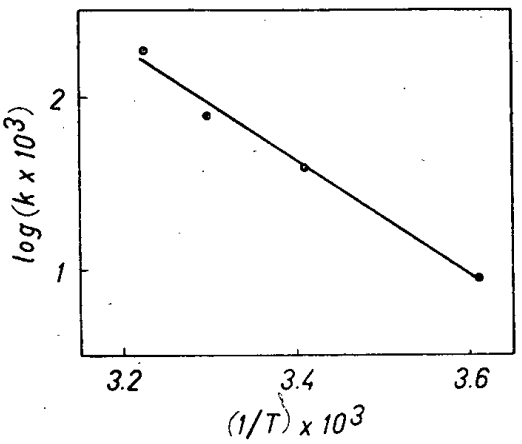

Fig. 5.
The temperature dependence of the inactivation rate
1M final concentration of $NaNO_2$, pH 5.55; temperatures: 4, 20, 30 and 37° C.

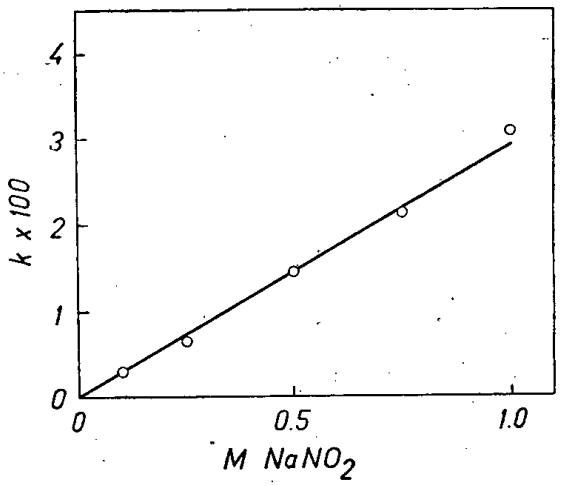

Fig. 6.
Dependenceof the inactivation rate on the concentration of $NaNO_2$
20 °C, pH 5.7.

Effect of pH and $NaNO_2$ concentration

According to the mass action law the dissociation of HNO_2 is characterized by the equation

$$pH - pK = \log [NaNO_2] - \log [HNO_2] \quad (3)$$

As the rate of inactivation should be proportional to the concentration of free HNO_2, it was expected that k will be proportional to the concentration of $NaNO_2$ and that the plot of log k against pH will give a linear relationship. As shown in Figs 6 and 7, the experimental data obtained fitted well the expected relationships. Below pH 4.8 and above pH 5.8 the rate of inactivation was, under the conditions given in Fig. 7, too high and too low, respectively, to be measured with sufficient accuracy.

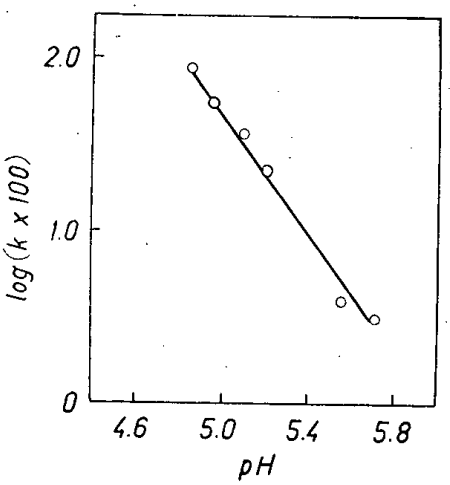

Fig. 7.

Dependence of the inactivation rate on pH
1M final concentration of $NaNO_2$, 20° C.

Attempt to isolate infectious DNA from Aujeszky disease virus

It was suspected that the shoulder in some inactivation curves was caused by the disturbing effect of viral protein, and possibly also of other substances surrouding the viral DNA, on the interaction of HNO_2 and the virus nucleic acid. Therefore it was attempted to isolate infectious DNA from the virus and to study its inactivation by HNO_2. However, all attempts to obtain infectious nucleic acid by treatment of crude or partially purified and concentrated virus preparations with cold or hot phenol were unsuccesful. The extracts showed no infectivity when tested in CEC tube cultures or monolayers using various isotonic and hypertonic NaCl and $MgSO_4$ solutions as diluents.

Immunogenicity of HNO_2-inactivated virus

Repeated intravenous injections of HNO_2-inactivated virus to rabbits in 1—2 ml. amounts led regularly to the development of virus neutralizing (VN) antibodies. The animals received 3—4 doses of inactivated virus at various intervals (see Fig. 8). At intervals, samples of blood were collected and the level of VN antibodies determined. The results of two representative immunisation experiments are presented in Fig. 8. The level of antibodies increased relatively slowly, reaching a maximum (VN titres 32—128) several weeks or even months after the last injection. In 2 out of 6 immunized rabbits the

antibodies persisted in relatively high levels for more than 3 months aft there last injection of inactivated virus, while in remaining animals antibodies either disappeared within 2 months or their level decreased to a minimum.

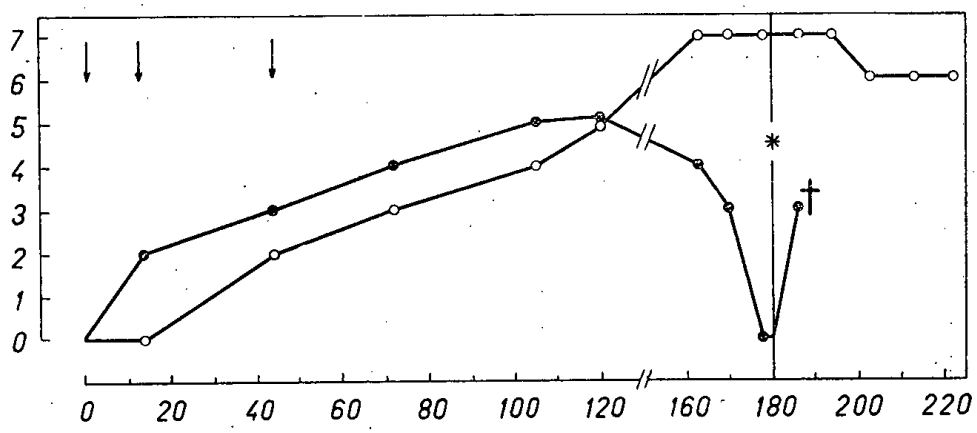

Fig. 8.

Immunisation of two rabbits with HNO_2 inactivated Aujeszky disease virus
abscissa: time in days; ordinate: VN titre (log_2 scale);
0 on the ordinate means no protection against the cytopathic effect of the virus; arrows indicate the time of injection of inactivated virus; * = intramuscular challenge with homologous live virus; † = death of the animal.

Animals vaccinated with the inactivated virus were protected against intramuscular challenge with 300 $TCID_{50}$ of homologous virus as long as antibodies were preserved (se eFig. 8). All nonimmune control animals infected with similar or even much lower doses of virus succumbed under typical symptoms of the disease.

Even repeated subcutaneous administration of the inactivated virus did not led to antibody formation in 4 rabbits. The fifth animal exhibited an extremely low level of VN antibodies after the third and fourth injections.

Discussion

In general, the kinetics of inactivation of Aujeszky disease virus by HNO_2 deviated from the strictly exponential inactivation observed with other viruses (Schuster and Schramm, 1958; Tessman, 1959, Schäfer *et al.*, 1959; Bautz-Freese and Freese, 1961; Restle *et al.*, 1962). The lines characterizing the dependence of log (I_0/I) on the time of inactivation were regularly shifted upwards (positive intercept). This fact can be interpreted in two different ways. Either an extremely rapid inactivation of an appreciable proportion of virus particles occurred immediately after exposure to HNO_2, which was then slowed down and proceeded further at a much lower rate as a first order reaction, or the reaction was not stopped completely immediately after dilution of the sample with phosphate buffer pH 7.85 and HNO_2 bound by or penetrated into the virus could react for an additional period of time with viral protein

and DNA. The former explanation seems to be more probable, since at relatively low temperatures or low HNO_2 concentrations this initial inactivation became somewhat slower and was manifested by a shoulder in the inactivation curve. Similar survival curves were observed in inactivation of poliovirus (Boeyé, 1959), bacterial transforming DNA (Litman and Ephrussi-Taylor, 1959; Stuy, 1962) and especially of type 1 adenovirus (Wassermann, 1962), indicating that at least two different components are involved in the inactivation by HNO_2. The possibility that the initial shoulder observed in some inactivation curves of Aujeszky disease virus was caused by the heterogeneity of virus population in respect to its sensitivity to HNO_2 must be rejected, because plaque purified virus displayed also under appropriate conditions a shoulder in the inactivation curve. It is highly probable that the action of HNO_2 on the infectivity of Aujeszky disease virus involved, as suggested also for other viruses (Boeyé, 1959, 1962; Restle *et al.*, 1962; Wassermann, 1962), both the viral DNA and protein. The fact that infectious RNA can be liberated from HNO_2-inactivated poliovirus (Boeyé, 1962) indicates that the alteration of the viral protein coat only may cause inactivation. It was also found that intact poliovirus was inactivated about three times faster by HNO_2 than its isolated infectious RNA (Boeyé, 1959). Thus the initial rapid inactivation observed in the reaction of HNO_2 with Aujeszky disease virus may correspond mainly to the alteration of viral protein and the slower exponential inactivation mainly to that of viral DNA. Aujeszky disease virus particles have a diameter of 1500—1800 Å (Reissig and Kaplan, 1962) and a 1 : 25 ratio of protein to DNA (Ben-Porat and Kaplan, 1962). To reach the viral DNA located inside of the virus particle, HNO_2 must penetrate trough the broad coat consisting of protein and possibly also of lipids and saccharides. Unfortunately we did not succeed in isolating infectious DNA from this virus and thus we could not compare the kinetics of inactivation by HNO_2 of intact virus and its DNA.

The velocity constants, the activation energy, the dependence of k on pH and $NaNO_2$ concentration determined in this study are all related to the second, exponential stage of inactivation. A linear dependence of the inactivation rate on H^+ ion concentration and its proportionality to $NaNO_2$ concentration was also found with T2 bacteriophage (Vielmetter and Wieder, 1959; Vielmetter and Schuster, 1960).

Aujeszky disease virus inactivated by HNO_2 retained at least a part of its original immunogenicity as manifested by the fact that rabbits immunized with killed virus developed specific antibodies and showed resistance against challenge with homologous live virus. Our samples of inactivated virus did not contain live virus in 2 ml. of suspension concentrated 10 times by sedimentation, because all animals survived the injection of the first dose of inactivated virus. Rabbits namely cannot survive infection even with extremely low doses of the virus strain used in this study. Repeated intravenous injections of the inactivated virus were necessary to obtain a relatively high level of antibodies in the blood and to ensure its persistence for a prolonged period of time. Further experiments are necessary to evaluate the efficiency of HNO_2-inactivated virus in vaccination. Poliovirus inactivated by HNO_2 to a sufficiently safe level was found to be of little if any value for immunisation. Its immunogenicity was

lost almost completely (Boeyé, 1962; Restle *et al.*, 1962). The latter finding cannot be generalized, however, for all viruses, as e.g. the composition and the structure of Aujeszky disease virus is quite different from that of poliovirus.

In plaque titrations of HNO_2-treated virus the occurrence of small-plaque-type mutants was regularly observed. The biological properties of the mutants and their sensitivity to HNO_2 treatment will be described later.

Summary

The kinetics of inactivation of Aujeszky disease virus by nitrous acid was investigated at various concentrations of sodium nitrite, pH of the reaction mixture and temperatures. After an initial short, but very rapid inactivation of an appreciable proportion of virus particles the reaction was slowed down and proceeded further with an exponential rate. For the exponential portion of the inactivation curves, the rate of inactivation was proportional to $NaNO_2$ concentration and linearly dependent on the hydrogen ion concentration. The activation energy of the reaction was 15.2 kcal/mole. Attempts to isolate infectious deoxyribonucleic acid (DNA) from the virus by phenol extraction were unsuccessful and thus the kinetics of inactivation of intact virus could not be compared with that of isolated DNA. It was concluded that the action of nitrous acid on the infectivity of the virus involved both viral DNA and protein. Virus inactivated by nitrous acid retained its immunogenicity. Rabbits immunized with the killed virus developed specific virus neutralizing antibodies and showed resistance to challenge with homologous live virus.

References

Bautz-Freese, E., and Freese, E. (1961): Induction of reverse mutations and cross reactivation of nitrous acid-treated phage T4. *Virology* **13,** 19.

Ben-Porat, T., and Kaplan, A. S. (1962): The chemical composition of herpes simplex and pseudorabies viruses. *Virology* **16,** 261.

Boeyé, A. (1959): Induction of a mutation in poliovirus by nitrous acid. *Virology* **9,** 691.

Boeyé, A. (1962): Inactivation of protein in poliovirus by nitrous acid. *Nature (Lond.)* **193,** 601.

Carp, R. I., and Koprowski, H. (1962): Mutation of type 3 poliovirus with nitrous acid. *Virology* **17,** 99.

Granoff, A. (1961): Induction of Newcastle disease virus mutants with nitrous acid. *Virology* **13,** 402.

Litman, R. M., and Ephrussi-Taylor, H. (1959): Inactivation et mutation des facteurs génétiques de l'acide desoxyribonucléique du pneumocoque par l'ultraviolet et par l'acide nitreux. *C. R. Acad. Sci. (Paris)* **249,** 838.

Mundry, K. W., and Gierer, A. (1958): Die Erzeugung von Mutationen des Tabakmosaikvirus durch chemische Behandlung seiner Nucleinsäure *in vitro*. *Z. Vererbungslehre* **89,** 614.

Reissig, M., and Kaplan, A. S. (1962): The morphology of noninfective pseudorabies virus produced by cells treated with 5-fluorouracil. *Virology* **16,** 1.

Restle, H., Hennessen, W., and Schäfer, W. (1962): Verhalten der antigenen und sonstigen biologischen Eigenschaften menschen- und tierpathogener Virusarten beim Behandeln mit salpetriger Säure 1. Mitt.: Verhalten des Poliomyelitis-Virus. *Z. Naturforsch.* **17***b*, 228.

Schäfer, W., Zimmermann, T., and Schuster, H. (1959): Inaktivierung verschiedener menschen- und tierpathogener Virusarten sowie des Tabakmosaik-Virus durch salpetrige Säure. *Z. Naturforsch.* **14***b*, 632.

Schuster, H., and Schramm, G. (1958): Bestimmung der biologisch wichtigen Einheit in der Ribosenucleinsäure des TMV auf chemischem Wege. *Z. Naturforsch.* **13***b*, 697.

Stuy, J. H. (1962): Inactivation of transforming deoxyribonucleic acid by nitrous acid. *Biochem. biophys. Res. Commun.* **6,** 328.

Škoda, R., and Mayer, V. (1961): Über die Züchtung des Virus der Aujeszkyschen Krankheit in einschichtigen Hühnerembryo-Fibroblastenkulturen mittels der Plaquemethode. *Arch. exp. Vet.-Med.* **15,** 391.

Tessman, I. (1959): Mutagenesis in phages ΦX174 and T4 and properties of the genetic material. *Virology* **9,** 375.

Vielmetter, W., and Schuster, H. (1960): Die Basenspezifität bei der Induktion von Mutationen durch salpetrige Säure im Phagen T2 *Z. Naturforsch.* **15b,** 304.

Vielmetter, W., and Wieder, C. M. (1959): Mutagene und inaktivierende Wirkung salpetriger Säure auf freie Partikel des Phagen T2. *Z. Naturforsch.* **14b,** 312.

Wassermann, F. E. (1962): The inactivation of adenoviruses by ultraviolet irradiation and nitrous acid. *Virology* **17,** 335.

Acta virol. **7** : 16—24, 1963

Mutations of Virulent and Attenuated Poliovirus Strains Induced by Nitrous Acid

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Received September 4, 1962

Recent investigations have shown that mutants exhibiting new biological properties can be obtained by direct treatment of viral nucleic acid with mutagenic agents capable to alter the arrangement of nucleotides (Gierer and Mundry, 1958; Schuster and Schramm, 1958; Siegel, 1960; Fraenkel-Conrat and Tsugita, 1961).

In the present study mutations occurring in virulent and attenuated poliovirus strains under the action of nitrous acid (HNO_2), capable to cause desamination of purine (adenine, guanine) and pyrimidine (cytosine) bases of viral ribonucleic acid (RNA) (Schuster and Schramm, 1958; Schuster *et al.*, 1960), were investigated.

Materials and Methods

Viruses. The virulent type 1, Mahoney, and type 2, MEF_1, poliovirus strains and Sabin's type 1, LSc 2ab, and type 2, P-712 Ch 2ab attenuated vaccine strains were used. Genetically homogenous lines of these viruses, isolated from plaques formed after infection with viral RNA, were used throughout. By this method virus lines with a high degree of homogeneity of the genetic markers can be obtained (Ghendon *et al.*, 1961*a*).

Tissue culture. Primary cultures of *Macaca rhesus* monkey kidney cells, obtained by dispersion of the tissue by trypsin, were used.

Isolation of viral RNA. The phenol extraction method of Gierer and Schramm (1956) was used.

Treatment with HNO_2. Both intact virus and isolated viral RNA were treated with HNO_2. Two volumes of the preparation examined were mixed with one volume of acetate buffer pH 3.3, 4.2, 4.7 or 5.2, and one volume of 4M $NaNO_2$. The reaction was allowed to proceed in rubber stoppered tubes at room temperature. The tubes were not agitated during the reaction. Separate tubes were used for each time interval. The reaction was stopped by diluting the mixture 1 : 10 with 0.02 M phosphate buffered 1.2M saline pH 7.8, after which 0.2 ml. portions of the material examined were inoculated into monkey kidney cell monolayers washed twice with phosphate buffer. Viral RNA and intact virus were allowed to adsorb at 37° C for 30 or 60 minutes, respectively, and the monolayers were then overlaid with agar medium prepared as described by Hsiung and Melnick (1957). In several experiments bottle cultures of monkey kidney cells in medium 199 were inoculated with 1.0 ml of a 1 : 10 dilution of the sample examined.

Isolation of mutants. Mutations of virulent poliovirus strains were investigated in cultures overlaid with agar medium and incubated at 36° C. Mutations of attenuated poliovirus strains were studied in two types of culture: (1) infected bottle cultures in liquid medium 199 were incubated in parallel at 36° and 40° C and examined for 7 days; cultures showing a characteristic cytopathic effect were investigated further for the genetic markers of the virus; and (2) cultures overlaid with agar were incubated at 36° C, while parallel bottles were incubated first for 30 hours at 40° C and then at 36° C. This procedure facilitated the isolation of mutants with S^+T^+ genetis markers. After plaques had developed in cultures infected with virulent or attenuated strains, their size was determined and from all isolated plaques virus clones were subcultured by a method described previously (Ghendon *et al.*, 1961*a*) The genetic markers of the virus clones obtained were then investigated.

Genetic markers. The folowing markers were studied: N — neurovirulence for monkeys after intracerebral inoculation; M — the character of morphological changes in the central nervous system of infected monkeys; mN — neurovirulence for mice after intracerebral inoculation;

T (rct_{40}) — ability to multiply at 40° C; d — ability to multiply at low sodium bicarbonate concentration; S — plaque size. The methods used in studying these markers were described previously (Ghendon *et al.*, 1961*b*).

Experiments on attenuated and virulent strains were carried out in separate rooms isolated from one another.

The data given in the figures and tables represent means from 3—4 experiments done at different times.

Results

First, the kinetics of inactivation by HNO_2 of intact virulent or attenuated polioviruses and of infectious RNA isolated from them were investigated. The results obtained showed that the rate of inactivation increased with decreasing the pH of the reaction mixture. At any pH the intact virus was inactivated more rapidly than the corresponding infectious RNA. There was no difference between attenuated and virulent polioviruses in the rate of their inactivation by HNO_2, irrespective of whether intact virus or infectious RNA were subjected to HNO_2 treatment. In control experiments the preparations examined were exposed to acetate buffers without the addition of $NaNO_2$. Neither intact virus nor infectious RNA were inactivated under these conditions within the reaction time used (2—5 minutes at pH 3.3 or 4.2; 20 minutes at pH 4.7; and 60 minutes at pH 5.2).

As next, we studied the appearance of mutants in virulent type 1 poliovirus, strain Mahoney, following treatment of intact virus or infectious RNA with HNO_2. The alteration of the T(rct_{40}) genetic marker served as the basic criterion of mutation in these experiments. (Virulent poliovirus strains with a T^+ genetic marker multiply equally well at 36° and 40° C, while attenuated strains with a T- genetic marker do not multiply at 40° C.)

Data presented in Fig. 1 show that the number of mutants increased with prolonging the time of treatment and, as the mutation rate is closely connected with the rate of deamination of nucleotides, with lowering the pH of the reaction mixture. No T- mutants were observed in control preparations of

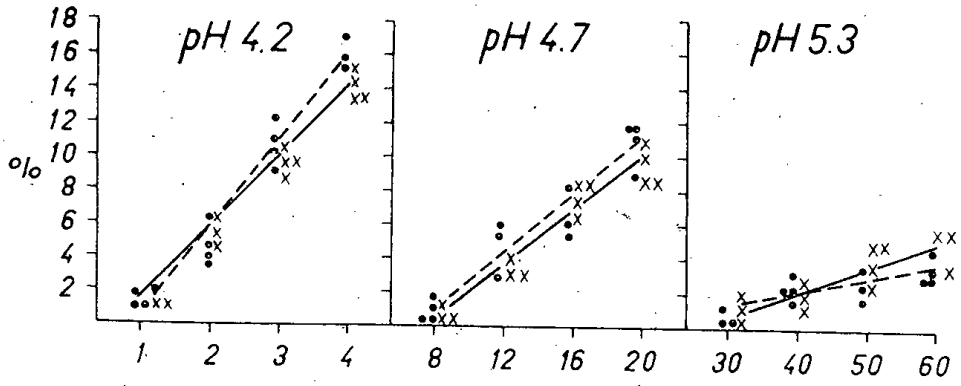


Fig. 1.

Induction of T- mutants by treatment of the Mahoney virulent poliovirus strain with HNO_2 in dependence on the reaction time and pH of the medium

● - - - - ● isolated viral RNA; × ——— × intact virus

Abscissa: time of treatment in minutes; ordinate: % of mutants among survivors.

ntact virus or infectious RNA exposed only to acetate buffer pH 4.2, 4.7 or 5.3 for 4, 20 and 60 minutes, respectively.

Further experiments were devoted to mutations of attenuated type 1

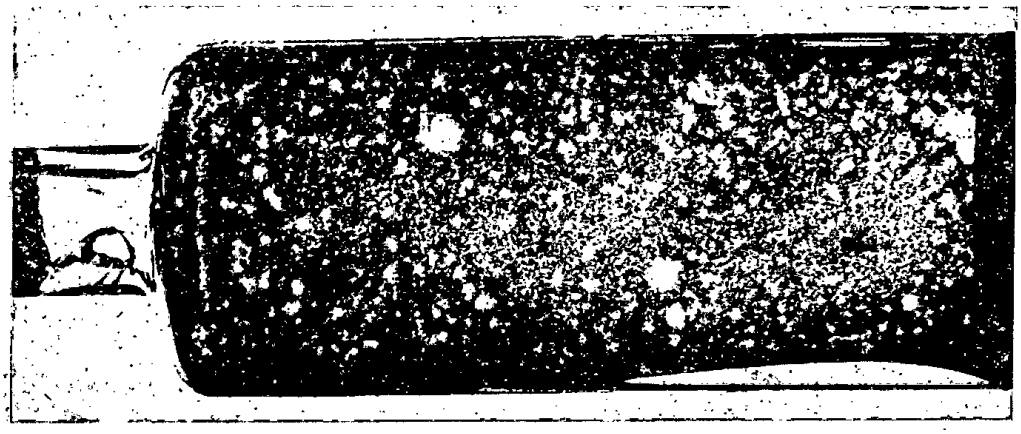
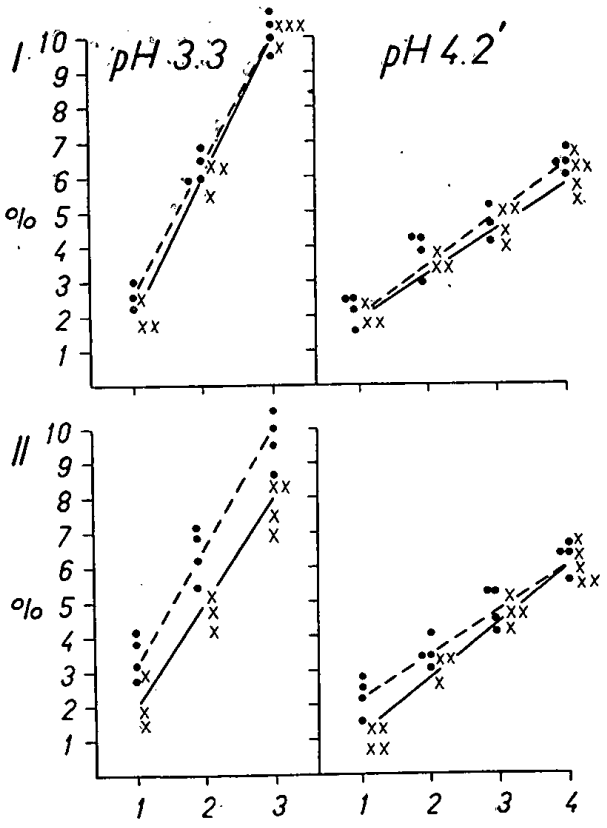


Fig. 2:

N^+ mutants induced by treatment of the attenuated LSc 2ab strain of type 1 poliovirus with HNO_2 Sote the single large S^+ plaques on the background of small S^- plaques.



poliovirus strain LSc 2ab, induced by treatment with HNO_2. First, monkey kidney cell cultures were inoculated with nitrous acid-treated virus and, after adding liquid nutrient medium, incubated at 40° C. In this way optimal conditions for the growth of T^+ mutants were ensured, if such mutants were formed following treatment with HNO_2. Results of these experiments showed that treatment of attenuated poliovirus for only a few minutes was sufficient to induce mutants capable to multiply at 40° C (T^+ mutants).

Fig. 3.

Frequency of S^+ and T^+ mutants among surviving virus following treatment of attenuated poliovirus with HNO_2
I — isolated viral RNA; II — intact virus: ●- - - - ● S^+ mutants; ×——× T^+ mutants. Abscissae and ordinates as in Fig. 1.

Then a quantitative study of the formation of S^+ and T^+ mutuants following treatment of attenuated poliovirus with HNO_2 was carried out. Tissue cultures were inoculated with nitrous acid-treated preparations of intact virus or infectious RNA, overlaid with agar medium and incubated first for 30 hours at 40° C and then at 36° C. These conditions favoured the multiplication of S^+ clones, which could be clearly differentiated from S^- clones (see Fig. 2). It is evident from the results summarized in Fig. 3 that treatment of attenuated virus with HNO_2 led regularly to the appearance of S^+ and T^+ mutants. As with virulent poliovirus strains, the number of these mutants increased by prolonging the time of treatment or by lowering the pH of the reaction mixture.

The genetic markers of several mutants obtained were investigated in detail in special experiments. The properties of the mutants showing various combinations of genetic markers are shown in Table 1. In addition to changes of the S and T genetic markers also other properties changed of both virulent and attenuated strains. With several mutants there occurred a separation of

Table 1. Genetic markers of poliovirus mutants induced by nitrous acid

Virus strain	Mutant	Genetic markers					
		N	M	T	d	S	mN
Mahoney, type 1	Original strain	+	+	+	+	+	
	M/4.2/3	+	+	+	+	—	
	M/4.7/4	+	+	—	—	—	
	M/4.2/6	—	—	+	+	+	
	M/4.2/2	—	—	—	—	—	
MEF_1, type 2	Original strain	+	+	+	+	+	+
	MF/4.2/1	+	+	—	+	—	+
	MF/4.2/9	+	+	—	—	—	+
	MF/4.7/6	—	—	+	+	+	+
	MF/4.2/2	—	—	—	—	—	+
	MF/4.2/5	—	—	+	+	+	—
	MF/4.2/3	—	—	—	—	—	—
LSc 2ab, type 1	Original strain	—	—	—	—	—	
	L/3.3/2	—	—	—	—	+	
	L/3.2/5	—	—	+	+	+	
	L/3.3/3	+	+	+	+	+	

+ and — = genetic markers characteristic of virulent and attenuated strains, respectively

very closely related genetic markers, as of the virulence for mice and monkeys (mutants MF/4.7/6 and MF/4.2/2), or of the T, d, and S genetic markers (mutants M/4.2/3, MF/4.2/1 and L/3.3/2). Other mutants exhibited changes in all the genetic markers examined. Mutants with genetic characters of attenuated strains, including apathogenicity for monkeys (mutants M/4.2/2 and MF/4.2/3), were obtained from virulent poliovirus strains, and a mutant

Table 2. The stability of genetic markers of poliovirus mutants in the course of passaging in tissue cultures

Original strain and its genetic markers	Mutant	Changes of genetic markers in the course of passaging			
		1st passage	2nd passage	3rd passage	4th passage
Mahoney N+M +T+d+S+	M/4.2/1	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—
	M/4.2/2	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—
	M/4.2/4	N—M—T—d—S—	N+M+T+d+S+	N+M+T+d+S+	N+M+T+d+S+
	M/4.7/8	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—
	M/4.7/12	N—M—T—d—S—	N—M—T—d—S—	N—M—T—D—S—	N—M—T—d—S—
	M/5.2/40	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—
	M/5.2/50	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—	N—M—T—d—S—
MEF$_1$ N+M+ T+d+ S+mN+	MF/4.2/3	N—M—T—d—S—mN—	N—M—T—d—S—mN—	N—M—T—d—S—mN—	N—M—T—d—S—mN—
	MF/4.2/9	N+M+T—d—S—mN+	N+M+T—d—S—mN+	N+M+T—d—S—mN+	N+M+T—d—S—mN+
	MF/4.2/5	N—M—T+d+S+mN—	N—M—T+d+S+mN—	N—M—T+d+mN—	N—M—T+d+S+mN—
L.Sc 2ab N—M—T— d—S—	L/4.2/1	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.2/2	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.2/31	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.2/32	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.2/61	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.2/62	T—d—S—	T—d—S—	T—d—S—	T—d—S—
	L/4.7/20	T—d—S—	T—d—S—	T—d—S—	T—d—S—

with characters of a virulent virus (L/3.3/3) was obtained from the attenuated strain.

The stability of the newly acquired genetic markers of several mutants during passaging in tissue cultures was also investigated. The results obtained (Table 2) showed that the genetic markers of the prevailing proportion of the mutants examined remained completely stable on passaging in tissue culture.

The results obtained by Mundry (1959), Boeyé (1959), Vielmetter and Wieder (1959) and Siegel (1960) ruled out the suggestion by Bawden (1959) that treatment of viruses with HNO_2 does not induce mutations, but leads to selection of particles less sensitive to the inactivating action of HNO_2. Never-

Table 3. Isolation of virus clones from mixtures of virulent and attenuated poliovirus strains treated with nitrous acid

Mixture	Treatment	Number of clones examined	Number of clones with genetic markers		Significance
			T+S+	T—S—	
97% Mahoney + 3% LSc 2ab, intact virus	untreated	252	244	8(3.2%)	$\chi^2 = 0.024$
	pH 4.2, 2 mins.	174	168	6(3.5%)	$P > 1.0$
97% Mahoney + 3% LSc 2ab RNA	untreated	116	112	4(3.5%)	$\chi^2 = 0.12$
	pH 4.2, 4 mins.	112	109	3(2.7%)	$P > 1.0$
97% LSc 2ab + 3% Mahoney intact virus	untreated	133	4(3.0%)	129	$\chi^2 = 0.018$
	pH 4.2, 2 mins.	108	3(2.8%)	105	$P > 1.0$
97% LSc 2ab + 3% Mahoney RNA	untreated	121	4(3.3%)	117	$\chi^2 = 0.067$
	pH 4.2, 4 mins.	127	5(4.1%)	122	$P > 1.0$

theless we carried out experiments to prove once more that the appearance of virus clones with unusual genetic markers after treatment of poliovirus with HNO_2 is a result of mutation and not of selection. Known amounts of virulent and attenuated polioviruses were mixed and treated with HNO_2, and the S and T markers of the viruses before and after treatment were determined. The data presented in Table 3 show that the ratio of virulent to attenuated viruses did not change after treatment of the mixture with HNO_2, as the differences observed were not statistically significant. The results of this experiment, together with the aforementioned data on the same rate of inactivation by HNO_2 of both virulent and attenuated poliovirus strains, confirmed the finding by other investigators that the appearance of virus clones with altered genetic markers in the viral population surviving after HNO_2 treatment is a result of mutation and not of selection resulting from

a different sensitivity to HNO_2 of virus particles showing different genetic markers.

Discussion

In the present experiments mainly the virulent strain Mahoney and the attenuated strain LSc 2ab of type 1 poliovirus were used in studying mutations induced by the action of HNO_2. The virus strains used were found previously to be genetically highly homogeneous (Ghendon *et al.*, 1961*b*; Ghendon and Diskina, 1962). The present investigations showed that treatment with HNO_2 of both virulent and attenuated poliovirus strains led to the appearance of mutants. Bautz-Freese and Freese (1961) reported that reversion of genetic markers may occur on treatment of some mutants with HNO_2. However, as shown by Wittmann (1961), such reversion can take place only with mutants obtained by the action of mutagens other than HNO_2. In agreement with the latter finding we did not observe reversion of genetic markers following treatment with HNO_2 of mutants induced by HNO_2 and possessing properties of attenuated strains (Ghendon, unpublished). On the other hand, treatment with HNO_2 regularly induced mutations of attenuated strains selected by Sabin. These findings suggest that changes of biological properties of poliovirus may be related with different mechanisms affecting the nucleotide composition of the viral nucleic acid.

Studies on Newcastle disease virus (Granoff, 1961) and bacteriophages (Freese, 1959; Vielmetter and Schuster, 1960) showed that the frequency of nitrous acid-induced mutants increased with lowering the pH of the reaction mixture. Vielmetter and Schuster (1960) found with T2 bacteriophage that increasing the pH of the medium up to 5.0 decreased more markedly the rate of mutation than that of inactivation by HNO_2. The results of our experiments on poliovirus confirmed that the frequency of mutants induced by HNO_2 increased with lowering the pH of the reaction mixture. However, the pH dependence of the inactivation rate was the same as that of mutation rate, i.e. both processes had a parallel course at any pH examined. This fact suggested that the ratios of the deamination rates of the nucleotides in poliovirus RNA, in contrast to the phage DNA (Vielmetter and Schuster, 1960), remained constant and were independent of the pH at which RNA was treated with HNO_2.

Our experiments showed that, if using HNO_2 as mutagen, mutants can be obtained on treatment of both infectious RNA and intact virus. But the proportion of mutants among survivors was much higher with isolated viral RNA than with intact virus. This was apparently caused by the fact that, in addition to deamination of bases of the viral RNA, also alterations of the protein coat may lead to the inactivation of intact virus. Thus in experiments aimed at obtaining mutants it is more advantageous to treat directly the carrier of genetic informations, the naked viral RNA, than the corresponding intact virus. When exposing isolated RNA to the action of HNO_2, inactivation due to deamination of the viral protein coat is avoided and optimal conditions

for the induction of mutations can be achieved, offering the possibility of obtaining great numbers of mutants.

Summary

1. The rate of poliovirus inactivation by nitrous acid increased with lowering the pH of the reaction mixture. Intact viruses were inactivated at a higher rate than the corresponding infectious viral ribonucleic acids (RNA). There was no difference between virulent and attenuated poliovirus strains in the rate of their inactivation by nitrous acid.

2. Treatment with nitrous acid of infectious RNA derived from virulent poliovirus strains led regularly to the appearance of T^- mutants among surviving virus. The number of mutants increased with prolonging the time of treatment or with lowering the pH of the reaction mixture. The ratio of the inactivation rate to the rate of mutation was independent of the pH at which the reaction proceeded.

3. A part of the mutants obtained by treatment of attenuated poliovirus strains with nitrous acid possessed all genetic markers typical of virulent strains. As with virulent strains, the frequency of mutation increased with prolonging the reaction time or with lowering the pH of the medium.

4. When treating attenuated or virulent intact viruses instead of isolated viral RNA with HNO_2, mutants were also obtained, but the ratio of the rate of mutation to the inactivation rate was considerably less than in experiments on viral RNA.

5. Mutants obtained after treatment of virulent and attenuated poliovirus strains with nitrous acid exhibited various combinations of genetic markers. Mutants obtained after treatment of virulent strains and possessing all the genetic markers characteristic of attenuated strains could be of practical importance.

References

Bawden, F. (1959): Effect of nitrous acid on tobacco mosaic virus: mutation or selection? *Nature (Lond.)* **184,** 27—29.

Bautz-Freese, E., and Freese, E. (1961): Induction of reverse mutations and cross reactivation of nitrous acid-treated phage T 4. *Virology* **13,** 19—30.

Boeyé, A. (1959): Induction of a mutation in poliovirus by nitrous acid. *Virology* **9,** 691—700.

Fraenkel-Conrat, H., and Tsugita, A. (1961): Effect of chemical alteration of RNA of tobacco mosaic virus on the structure of protein and biological properties. 5th Int. Congr. Biochem., Moscow, 1961, Symposium 3, Evolutionary biochemistry **3,** 19—20.

Freese, E. (1959): On the molecular explanation of spontaneous and induced mutations. *Brookhaven Symposia in Biology* **12,** 63—75.

Ghendon, Yu. Z., and Diskina, B. S. (1962): Vaccine strains of poliovirus obtained by different physical treatments of ribonucleic acid isolated from virulent strains. *Acta virol.* **6,** 289—296

Ghendon, Yu. Z., Diskina, B. S., and Marchenko, A. T. (1961*a*): Infection of tissue cultures with viral RNA as a method for isolation of virus clones with stable genetic markers. *Vop. Virusol.* **6,** 651—656 (in Russian).

Ghendon, Yu. Z., Khesin, Ya. E., and Marchenko, A. T. (1961*b*): Studies on the stability of genetic markers of Sabin's attenuated poliovirus strains. In: *Oral live vaccine against poliomyelitis,* 461—483 (in Russian).

Gierer, A., and Mundry, K. (1958): Production of mutants of tobacco mosaic virus by chemical alteration of its ribonucleic acid *in vitro*. *Nature (Lond.)* **182,** 1457—1458.

Gierer, A., and Schramm, G. (1956): Infectivity of ribonucleic acid from tobacco mosaic virus. *Nature (Lond.)* **177,** 702—703.

Granoff, A. (1961): Induction of Newcastle disease virus mutants with nitrous acid. *Virology* **13,** 402—408.

Hsiung, G., and Melnick, J. (1957): Morphologic characteristics of plaques produced on monkey kidney monolayer cultures by enteric viruses (poliomyelitis, coxsackie and ECHO groups). *J. Immunol.* **78,** 128—136.

Mundry, K. (1959): The effect nitrous acid on tobacco mosaic virus: mutation, not selection. *Virology* **9,** 722—726.

Schuster, G., Gierer, A., and Mundry, K. (1960): Inaktivierende und mutagene Wirkung der chemischen Veränderung von Nucleotiden in Virus Nucleinsäure. *Abhandl. Dtsch. Ak. Wiss. Berlin. Kl. Med.* **1,** 76—85.

Schuster, H., and Schramm, G. (1958): Bestimmung der biologisch wirksamen Einheit in der Ribosenucleinsäure des Tabakmosaikvirus aur chemischem Wege. *Z. Naturforsch.* **13b,** 697—704.

Siegel, A. (1960): Studies on the induction of tobacco mosaic virus mutants with nitrous acid. *Virology* **11,** 156—167.

Vielmetter, W., and Schuster, H. (1960): The base specificity of mutation induced by nitrous acid in phage T2. *Bioch. Bioph. Res. Com.* **2,** 324—328.

Vielmetter, W., and Wieder, C. (1959): Mutagene und inaktivierende Wirkung Salpetriger Säure auf freie Partikel des Phagen T2. *Z. Naturforsch.* **14b,** 312.

Wittmann, H. G. (1961): Studies on the nucleic acid-protein correlation in tobacco mosaic virus. 5th Int. Congr. Biochem., Moscow 1961, Symposium 1, Biological structure and fractions at the molecular level **6,** 39—44.

Acta virol. 7 : 25—36, 1963

Cofactor and Specific Antibodies against Influenza Viruses

VII. The Nature of Cofactor and Influenza Antibodies Studied by Density Gradient Zonal Centrifugation

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Received July 10, 1962

Based on pilot immunoelectrophoretic investigations we suggested that cofactor — a component of normal animal sera potentiating the effect of influenza antibodies — is of macroglobulin nature (Hána *et al.*, 1961). This assumption has been supported by the results of rivanol precipitation of mouse sera (Styk *et al.*, 1962b).

But we also showed (Styk *et al.*, 1962b) that cofactor occurs in sera from newborn pigs which had not yet been suckled. As pig placenta is impermeable for serum proteins originating from the mother (Brambell *et al.*, 1951; Brambell, 1958), serum from newborn unsuckled pigglets contains only proteins synthetized by the newborn organism. It was found (Šterzl *et al.*, 1960; Franěk *et al.*, 1961) that sera from newborn pigglets do not contain either 19S gamma-globulin ($beta_{2M}$-globulin) or 7S gamma-globulin. Such sera contain in the gamma-globulin region only components with a sedimentation coefficient of 3—6S (Franěk *et al.*, 1961) showing no antibody activity even against antigens to which adult pigs regularly possess the so-called natural antibodies. This fact has been confirmed by the extremely sensitive bactericidal test (Šterzl *et al.*, 1962). Thus the question arose as to the validity of our assumption concerning the macroglobulin character of cofactor. Two explanations of the discrepancy mentioned appeared possible: either sera from newborn pigs do contain macroglobulins or the nature of cofactor from such sera differs from the nature of cofactor from other animal sera.

In order to elucidate this question as well as the nature of cofactor from different animal sera we used density gradient zonal centrifugation, which method has been suggested for differentiating serum proteins of a different molecular weight (Edelman *et al.*, 1958). In a part of the experiments this method was supplemented by our modification of rivanol precipitation of serum (Hána and Styk, 1962*a*) which makes possible a partial separation of macroglobulins from other serum proteins.

These two methods were also used in investigating another problem. In a previous paper we reported about the different character of influenza antibodies from early immune and hyperimmune sera. The principal criterion for their differentiation — in addition to different sensitivities to inhibitor-destroying substances and to heating — was their different ability to be potentiated by cofactor (Styk, 1962). We are reporting the results of experiments, in which we studied by density gradient zonal centrifugation (Edelman *et al.*, 1958) whether the two kinds of antibody differ in their molecular weihgt.

Antibodies of different molecular weights are namely known to occur in man, rabbit, horse and pig (Kuhns, 1955; Stelos and Talmage, 1957; Kabat, 1943; Bauer and Stavitski, 1961; Franěk *et al.*, 1962; and others). In mice, different molecular weight antibodies have not yet been reported.

A brief preliminary account has been published (Styk *et al.*, 1962*a*).

Materials and Methods

Normal animal sera were kept frozen until used. Sera from white mice were pooled, those from other animals were examined individually. In the case of pigglets occasionally serum pools from two animals of the same litter were used. For details on the sera from newborn pigs see Styk *et al.* (1962*b*).

Early immune and hyperimmune sera were prepared in white mice as described previously (Styk, 1962). As antigens for immunization and in serological tests we used influenza virus strains A2/Bratislava/4/57 and B-Lee, both in the form of infective allantoic fluids. (In a part of the experiments viruses partially purified by adsorption onto and elution from formolized erythrocytes were used.)

Saccharose gradient zonal centrifugation. The method of Edelman *et al.* (1958) as modified by Říha (1963) was used. A discontinuous saccharose gradient in 0.15 M NaCl was prepared in lusteroid tubes of the 40.2 rotor of model L Spinco ultracentrifuge. The saccharose concentration was 40 and 10% at the bottom and top, respectively. The sera examined were mixed with an equal volume of 0.15 M NaCl solution and layered on the top of the gradient. After centrifugation at 32 000 rev/min. for 16 hours at 10° C the contents of the tubes were consecutively withdrawn by means of a hypodermic needle and syringe in six equal portions, starting from the top. The individual fractions were numbered I—VI from the top to the bottom. The cofactor contents of the fractions were determined after removing saccharose by dialysis. In a part of the experiments also the protein content of the fractions was estimated. Under these conditions 7S gammaglobulins were located in fractions II and III, macroglobulins in fractions IV and V, occasionally VI.

Rivanol precipitation was done as described by Hána and Styk (1962*a*). A 0.06% rivanol concentration was used which, according to our experiences, leads to the most selective precipitation of cofactor from mouse serum.

Cofactor titration was carried out by the method of Styk (1961) using detection sera anti-A2 (with non-avid influenza virus A2/Bratislava/4/57) or anti-B (with influenza virus B-Lee). The distribution of cofactor activity determined by these two sera was the same (with the exception of guinea pig serum in Table 1), though the absolute titre values determined using the anti-B serum were regularly lower. If not stated otherwise, the results given in the Tables are those obtained in titrations using anti-A2 detection serum.

Results

1. The nature of cofactor in different normal animal sera as revealed by saccharose density gradient zonal centrifugation

Table 1 presents the results concerning the distribution of cofactor activity in different normal animal sera after centrifugation in saccharose gradients. In mouse, bovine and rat sera maximal cofactor activity occurred in fractions IV and V. With two guinea pig sera the cofactor activity was found in several fractions when anti-A2 detection serum was used; when using anti-B dectection serum, the distribution of cofactor activity was narrower, but the titres were lower. With rat serum, the level of cofactor in fraction III could not be exactly determined because of haemolysins occurring in this fraction.

Sera from adult pigs (mothers) also showed maximal cofactor activities in fractions IV and V. A different behaviour was shown by cofactor in sera from

newborn unsuckled pigglets (Table 1). In all these sera examined maximal cofactor activity occurred in fractions I and II, which suggested that the molecular weight of cofactor in such sera was lower than in sera from adult

Table 1. Distribution of cofactor activity in different normal animal sera after saccharose gradient centrifugation

Fraction	Kind of serum										
	Mouse (Dobrá Voda breed)	Mouse (Děčín breed)	Mouse (3 weeks old mice)	Bovine	Rat	Guinea pig DS A2	Guinea pig DS B	Pig (mother No.1)	Pigglet of mother No. 1, before 1st feeding	Pig (mother No. 2)	Pigglet of mother No. 2, before 1st feeding
I	0	0	0	0	0	0	0	0	4	0	±2
II	0	0	0	±2	0	±4	0	1	16	0	4
III	0	0	0	±2	4 (Haem)	±4	0	2	4	0	±1
IV	1	2	4	8	±32	4	2	8	2	±4	±2
V	±8	4	4	4	±16	±4	1	4	1	±2	0
VI	1	±1	1	2	8	1	0	1	0	0	0
Whole serum	±80	40	40	160	±320	±160	20	±160	80	±80	20

0 means < 1

pigs or other animals. Sedimentation analysis of fractions I and II obtained after centrifugation of sera from newborn unsuckled pigglets revealed a single component, the $s_{20,w}$ of which varied in the individual experiments from 3.01 S to 4.03 S. The protein content of these fractions varied from 2.4—6.0 mg./ml.

After taking food (maternal milk) the distribution of cofactor activity after centrifugation in the density gradient changed (Table 2). In ten-days-old

Table 2. Distribution of cofactor activity in sera from young pigs and from their mother (on the day of parturition) after saccharose gradient centrifugation

Fraction	Newborn pig (before 1st feeding)	10 days' pig	20 days' pig	Mother
I	4	±1	0	0
II	8	2	1	1
III	±2	2	±1	±2
IV	±1	±16	8	±8
V	0	2	±4	4
VI	0	0	±1	±1
Whole serum	±80	±160	±80	±160

0 means < 1

pigglets cofactor activity was still present in fractions I and II, but its maximum clearly moved to fraction IV.

This picture became more clear when rivanol-precipitated sera from the pigglets were subjected to density gradient centrifugation (Table 3). In newborn unsuckled pigglets the maximum of cofactor activity occurred in fractions I and II and after rivanol precipitation the prevailing part of the activity re-

Table 3. Distribution of cofactor activity in sera from young pigs after rivanol precipitation and saccharose gradient centrifugation

Age of the pigs (days)	Fraction	Whole serum	Supernatant fluid after rivanol treatment	Sediment after rivanol treatment
0 (before 1st feeding)	I	±8	±8	0
	II	8	16	0
	III	±2	0	0
	IV	1	(±1)	0
	V	0	0	0
	VI	0	0	0
	Original sample	±160	±80	±5
4	I	1	1	0
	II	2	4	0
	III	1	1	0
	IV	16	0	2
	V	4	0	±1
	VI	0	0	0
	Original sample	80	±40	40
24	I	0	0	0
	II	±1	±1	0
	III	1	0	0
	IV	8	0	1
	V	2	0	±1
	VI	±1	0	0
	Original sample	±160	±10	20

0 means < 1

mained in the supernatant fluid. With a 4-days-old pigglet, maximal cofactor activity was found on examination of whole serum in fraction IV; fraction II contained only a little more cofactor than fractions I and III. After rivanol precipitation the picture became more clear: the supernatant fluid showed maximal cofactor activity in fraction II and the sediment in fraction IV. It can be assumed that in this case the cofactor found in the supernatant fluid was cofactor produced by the pigglet itself, whereas cofactor found in fraction IV was cofactor resorbed from maternal colostrum.

Already in previous experiments (Styk *et al.*, 1962*b*) we observed that precipit-

ation with 0.06% rivanol does not remove from mouse serum all cofactor, but that a small part of the activity remains in the supernatant fluid. We therefore attempted to show whether cofactor remaining after rivanol precipitation of mouse serum in the supernatant fluid is of different nature, i.e. whether its molecular weight is lower than that of the precipitated cofactor. Data presented in Table 4 show that this was not the case and that cofactor occurring in the supernatant fluid apparently also represents a macroglobulin.

All the experiments described were repeated several times with comparable results.

Experiments on saccharose gradient centrifugation of normal animal sera offered an indirect support of the view (Hána *et al.*, 1961; Hána and Styk, 1962*a*) that cofactor apparently is not of beta-lipoprotein nature (see Polyak

Table 4. Distribution of cofactor activity in normal mouse serum after precipitation with rivanol and saccharose gradient centrifugation

Fraction	Whole serum	Supernatant fluid after rivanol treatment	Sediment after rivanol treatment
I	±1	0	±1
II	0	0	0
III	0	0	0
IV	±2	±1	±8
V	4	±2	4
VI	±2	±1	4
Original sample	±80	10	±80

0 means < 1

et al., 1961; Polyak and Yabrov, 1961). Several of the sera examined contained considerable amounts of lipids, occurring after centrifugation in fraction I; but the cofactor activity moved to fractions IV and V. Only in sera from newborn unsuckled pigglets the cofactor activity occurred in fractions I and II. To test whether at least in these cases does the cofactor contain a lipoid component, several sera were delipidized with ether by the method of McFarlane as modified by Hána and Styk (1960). Delipidization was repeated up to three times.

It was found that delipidization of both sera from unsuckled newborn pigglets and bovine and mouse (normal and immune) sera lowered the cofactor titre not at all or maximally by one half of a dilution step in the serological test (e.g. from 80 to ±80). Thus the lipoid component does not play a role in cofactor activity. Naturally, cofactor can occur in the beta-lipoprotein fraction obtained by Cohn's fractionation, but this fraction contains a mixture of different substances. The lipoid component does not possess cofactor activity, nor does it affect this activity by eventually covering reactive centres on the surface

of cofactor, which should become manifest by an increase of cofactor titre after delipidization.

2. The nature of specific antibodies from early immune and hyperimmune sera as revealed by saccharose gradient zonal centrifugation

The distribution of specific antibodies was studied in samples both unheated (which thus contained own cofactor in whole serum or in some of its fractions) and heated at 56° C for 30 minutes, i.e. after destruction of own cofactor.

Table 5. Distribution of cofactor and specific antibodies from early immune anti-A2 mouse serum after rivanol precipitation and saccharose gradient centrifugation

	Fraction	Cofactor (DS:B-Lee)	Antibodies in samples unheated		heated at 56°/30 mins	
			NaCl*	NMS*	NaCl*	NMS*
Whole serum	I	0	0	0	0	0
	II	0	0	4	0	4
	III	0	0	16	0	16
	IV	2	2	4	0	4
	V	4	0	2	0	±2
	VI	±2	0	0	0	0
	Original serum	80	80	80	< 10	160
Supernatant fluid after rivanol treatment	I	0	0	0	0	0
	II	0	0	16	0	16
	III	0	±2	64	0	64
	IV	0	±2	32	0	32
	V	(±1)	0	±4	0	4
	VI	(±1)	0	2	0	2
	Original sample	10	40	320	±20	320
Sediment after rivanol treatment	I	0	0	0	0	0
	II	0	0	0	0	0
	III	0	0	8	0	8
	IV	±4	0	±2	0	0
	V	2	0	0	0	0
	VI	1	0	0	0	0
	Original sample	40	40	80	±10	40

* Examined in saline (NaCl) or normal mouse serum (NMS) diluted 1 : 15 (i.e. with cofactor added).

0 means < 1 (cofactor) or < 2 (antibodies)

In both cases all samples were examined in saline and in normal native mouse serum diluted 1 : 15 or 1 : 25, i.e. with cofactor added. In addition, in unheated samples we examined the distribution of the own cofactor of immune

serum using heterotypic detection serum (B-Lee detection serum with anti-A2 immune sera and *vice versa*).

The greatest attention was paid to early and hyperimmune anti-A2 sera. All experiments yielded a picture similar to that illustrated in Tables 5 and 6. Maximum of antibodies occurred in fraction III, occasionally also in fraction II. Thus antibodies from both kinds of immune serum apparently possess the character of 7S gamma-globulin. (Especially heated samples, diluted with

Table 7. Distribution of cofactor and specific antibodies from early immune and hyperimmune B-Lee antiserum after saccharose gradient centrifugation

	Fraction	Cofactor (DS:A2)	Antibodies in samples unheated		heated at 56° C/30 mins	
			NaCl*	NMS*	NaCl*	NMS*
Early immune serum B-Lee	I	0	0	0	0	0
	II	0	0	0	0	0
	III	0	0	2	0	2
	IV	0	0	0	0	0
	V	1	0	0	0	0
	VI	±2	0	0	0	0
	Whole serum	±20	40	80	<10	±40
Hyperimmune serum B-Lee	I	0	0	0	0	0
	II	0	16	±64	16	32
	III	0	32	64	64	±128
	IV	0	16	32	±16	±32
	V	1	0	±4	2	±4
	VI	±2	±2	2	0	2
	Whole serum	±40	640	1280	320	1280

* See Table 5. NMS was diluted 1 : 25.

saline and with cofactor added, should be taken into account. In unheated samples the results were affected, especially with whole serum, by the presence of own cofactor.)

In early serum, antibody activity occurred also in fraction IV (partially in fraction V, too); it was similar to that found in fraction II or, in the case of supernatant fluid after rivanol precipitation, even higher than that in fraction II. However, it would be premature to conclude from these results that antibodies from early serum possess a greater molecular weight than antibodies from hyperimmune sera, especially because of the ambiguous results of control sedimentation analysis. In these experiments, both in the individual fractions and original samples, the previously reported (Styk, 1962) difference between antibodies from the two kinds of immune serum regarding their ability to be potentiated by cofactor (increase in the titre of heated samples after the addition of cofactor) again became clearly evident.

Table 6. Distribution of cofactor and specific antibodies from hyperimmune anti-A2 mouse serum

	Fraction	Cofactor (DS:B-Lee)	Antibodies in samples unheated		heated at 56°/30 mins	
			NaCl*	NMS*	NaCl*	NMS*
Whole serum	I	0	0	0	0	0
	II	0	8	±16	±4	8
	III	0	8	±32	8	8
	IV	±1	4	4	±2	±2
	V	8	0	0	0	0
	VI	2	0	0	0	0
	Original serum	±160	160	320	80	160
Supernatant fluid after rivanol treatment	I	0	0	0	0	0
	II	0	4	8	2	8
	III	0	8	±16	±4	±4
	IV	0	0	±2	0	0
	V	0	0	0	0	0
	VI	0	0	0	0	0
	Original sample	5	80	160	40	±160
Sediment after rivanol treatment	I	0	0	0	0	0
	II	0	0	0	0	0
	III	0	(0)	±2	0	0
	IV	±1	0	0	0	0
	V	±1	0	0	0	0
	VI	0	0	0	0	0
	Original sample	40	±40	40	10	20

* For explanations see Table 5

In all the immune sera examined we studied the distribution of cofactor activity in unheated samples by means of heterotypic detection serum. The results confirmed the macroglobulin character of cofactor from mouse serum. It should be added that on examination of cofactor in native immune serum apparently only free cofactor is determined. A (probably minor) part of cofactor apparently is bound to antibodies of the immune serum tested (see Styk and Hána, 1962).

Some of the sera examined were treated before centrifugation by ether as described above to remove the effect of lipoid substances present in the sera. One of the results obtained is illustrated in Table 6. Delipidization did not substantially affect either the titres of cofactor and antibodies in the original serum, or the distribution of cofactor and antibody activities after centrifugation (or after preceding rivanol precipitation).

The results obtained with immune sera against type B influenza virus were similar to those obtained with anti-A2 immune sera. Table 7 shows the results

after rivanol precipitation (or delipidization with ether) and saccharose gradient centrifugation

	Fraction	Cofactor (DS:B-Lee)	Antibodies in samples unheated		heated at 56°/30 mins	
			NaCl*	NMS*	NaCl*	NMS*
Whole serum, delipidized with ether	I	0	0	0	0	0
	II	0	4	8	2	4
	III	0	4	8	±2	±4
	IV	0	0	0	0	0
	V	2	0	0	0	0
	VI	0	0	0	0	0
	Original sample	±80	160	160	40	80
Delipidized serum, supernatant fluid after rivanol treatment	I	0	0	0	0	0
	II	0	2	4	0	2
	III	0	4	±8	±2	±4
	IV	0	0	0	0	0
	V	0	0	0	0	0
	VI	0	0	0	0	0
	Original sample	5	80	80	40	80
Delipidized serum, sediment after rivanol treatment	I	0	0	0	0	0
	II	0	0	0	0	0
	III	0	0	0	0	0
	IV	0	0	0	0	0
	V	1	0	0	0	0
	VI	2	0	0	0	0
	Original sample	80	40	40	20	±40

obtained with one early and one hyperimmune B-Lee serum. These results also concur with the 7S gamma-globulin character of antibodies and with the macroglobulin nature of cofactor. (It must be added that the comparatively low titres occasionally obtained — e.g. with the early B-Lee serum in Table 7 — apparently were the consequence of the fact that some samples could be tested for the presence of cofactor and antibody only several days after centrifugation. Nor the 24 hours' dialysis, though carried out in the cold, and the transport of samples from Prague, where centrifugation was done, to Bratislava, did favourably affect the levels of the active components in the fractions.)

Discussion

The results of saccharose density gradient centrifugation experiments confirmed the assumption (Hána *et al.*, 1961; Hána and Styk, 1962*a*) that cofactor occurring in mouse, bovine, pig and other sera is of macroglobulin nature. With respect to its behaviour on immunoelectrophoresis (Hána *et al.*,

1961) and its mobility on paper electrophoresis (Styk and Hána, 1960), cofactor probably represents a beta$_2$-macroglobulin (designated also gamma$_1$-macroglobulin or 19S gamma-globulin).

The character of cofactor found in serum from newborn unsuckled pigglets, i.e. animals devoid of 19S gamma-globulin (Franěk *et al.*, 1961), is different. In such sera cofactor has a much lower molecular weight than in sera from adult pigs and other animals, probably even lower than the 7S gamma-globulin of adult animals. This view is supported by the values obtained on sedimentation analysis of some samples which, however, need not be related with the pigglet's cofactor. Although sedimentation analysis revealed but one component in these fractions, cofactor could have been present in them in amounts lower than demonstrable by sedimentation analysis (though sufficient to be demonstrated serologically) and the S values found could have been shown by another substance. The assumption that cofactor from sera of newborn unsuckled pigglets has a molecular weight lower than 7S gamma-globulin has been confirmed by chromatographic separation of such sera on Sephadex G-200 gel (Hána and Styk, 1962*b*). It remains obscure whether there is some relation between cofactor from sera of unsuckled pigglets and non-antibody gamma-globulin which in these animals possesses also a lower molecular weight than 7S gamma-globulin.

One interesting question, which could not yet been answered, remains: how does the cofactor's molecular weight change? Or, in other words, which is the fate of the low molecular weight pigglet's cofactor after macroglobulin cofactor from the colostrum had penetrated into the blood stream of the pigglet? Is its formation going on? Several hypotheses can be offered. (*1*) High molecular weight cofactor is formed by aggregation of molecules of the „small" (light) cofactor. (May be the aggregation actually is the consequence of the presence of resorbed macroglobulin cofactor.) This could mean that the macroglobulin cofactor itself would represent only an aggregate of minor particles. (*2*) Cofactor activity is shown by a small particle bound to the macroglobulin. In such a case it should be possible to remove the active particle from the macroglobulin carrier. (Our experiments on 2-mercaptoethanol treatment were as yet unsuccessful, probably because of the comparatively high lability of cofactor.) (*3*) A whole macroglobulin molecule is needed for the full cofactor activity. In the organism of suckling pigs the „large" cofactor is formed de novo, probably more intensively after resorption of macroglobulin cofactor from colostrum. The formation of „small" cofactor stops later entirely, or there occurs immediate aggregation of the prevailing part of it.

Several approaches would be possible in investigating these problems: to follow the formation of cofactor in suckling pigs by means of radioactive tracers; to follow the formation of cofactor in newborn animals (or foetuses before delivery) of species possessing a permeable placenta (by labelling with radioactive izotopes or by other methods); to follow the development of cofactor in animals of a systematic rank lower than are the mammals; etc. We hope to answer at least some of these questions by further investigations.

The results concerning the character of specific influenza antibodies can be briefly summarized as follows: in spite of certain signs of a different mobility

of antibodies from early and hyperimmune sera on density gradient centrifugation, we did not find any significant difference in the size of their molecules. We confirmed only the different character of antibodies regarding their ability to be potentiated by cofactor. We consider density gradient zonal centrifugation not sufficiently sensitive to differentiate the two kinds of antibody. Thus we shall try to separate them by other methods.

Summary

Using saccharose density gradient zonal centrifugation we found that cofactor potentiating the effect of specific influenza antibodies possesses in different normal animal sera the character of a macroglobulin. By contrast, in sera from newborn unsuckled pigglets cofactor occurred in the two uppermost fractions, from which it follows that its molecular weight was lower than in the other animals, probably even lower than that of 7S gamma-globulin, which was supported by sedimentation analysis. After feeding colostrum the pigglets contained in their serum detectable amounts of both light and macroglobulin cofactor. This picture becomes especially marked after precipitation of the sera with rivanol and subsequent gradient centrifugation. The possible explanations of the phenomena observed are discussed.

Investigations on the distribution of specific antibodies from early immune and hyperimmune A2 and B influenza antisera showed that, in spite of certain differences in their mobility, both kinds of antibody apparently are of a 7S gamma-globulin character. The different nature of these two kinds of antibody regarding their ability to be potentiated by cofactor, as well as the macroglobulin character of cofactor in immune mouse serum were confirmed.

After delipidization of the sera with ether the cofactor titre practically did not decrease. This fact offers evidence against the possibility that lipoid components of serum would play a role in cofactor activity.

References

Bauer, D. C., and Stavitski, A. B. (1961): On the different molecular forms of antibody synthesized by rabbits during the early response to a single injection of protein and cellular antigens. *Proc. Nat. Acad. Sci. (Wash.)* **47**, 1667—1680.

Brambell, F. W. R. (1958): The passive immunity of the young mammal. *Biol. Rev.* **33**, 488—531.

Brambell, F. W. R., Hemmings, W. A., and Henderson M. (1951): *Antibodies and embryos.* Brambell, Hemmings and Henderson, London.

Edelman, G. M., Kunkel, H. G., and Franklin, E. C. (1958): Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin. *J. exp. Med.* **108**, 105—120.

Franěk, F., Jouja, V., and Kostka, J. (1962): The nature of pig antibodies against Brucella suis. *Folia microbiol.* **7**, 1—11.

Franěk, F., Říha, I., and Šterzl, J. (1961): Characteristics of gamma globulin lacking antibody properties in newborn pigs. *Nature (Lond.)*, **189**, 1020—1022.

Hána, L., Križanová, O., Styk, B., and Sokol, F. (1961): Some data on the nature of the cofactor enhancing the activity of imperfect antibodies against A2 influenza virus strains. *Acta virol.* **5**, 325.

Hána, L., and Styk, B. (1960): Influence of delipidisation on the haemagglutination inhibiting activity of rat serum against influenza type C viruses. *Acta virol.* **4**, 392—393.

Hána, L., and Styk, B. (1962*a*): An attempt to differentiate beta-inhibitor from cofactor occurring in bovine serum. *Acta virol.* **6**, 77—83.

Hána, L., and Styk B. (1962*b*): Characterization of antibodies, cofactor and nonspecific viral inhibitors on Sephadex G-200. *Acta virol.* **6,** 479.

Kabat, E. A. (1943): Immunochemistry of the proteins. *J. Immunol.* **47,** 513—587.

Kuhns, W. J. (1955): Immunological properties of a form of non-precipitating Diphteria antitoxin which does not sensitize human skin. *J. Immunol.* **75,** 105—111.

Polyak, R. Ya., and Yabrov, A. A. (1961): On the biochemistry of the thermolabile factor of normal sera, activating theeffect of immune anti-influenza sera. *Vop. virusol.* **6,** 678—684 (in Russian).

Polyak, R. Ya., Yabrov, A. A., and Smorodintsev, A. A. (1961): Experimental data on the chemical nature of a nonspecific thermolabile component of normal sera which enhances the activity of influenza virus antisera. *Acta virol.* **5,** 261.

Říha, I., (1963): The formation of 19S and 7S antibodies in young rabbits. *Folia microbiol.* (in press).

Stelos, P., and Talmage, D. W. (1957): The separation by starch electrophoresis of two antibodies to sheep red cells differing in hemolytic efficiency. *J. inf. Dis.* **100,** 126—135.

Styk, B. (1961): Cofactor and specific antibodies against influenza viruses. I. Method of cofactor titration. Cofactor content of various animal sera. *Acta virol.* **5,** 334—341.

Styk, B. (1962): Cofactor and specific antibodies against influenza viruses. III. The potentiating effect of cofactor on specific antibodies of early immune and of hyperimmune sera and the differences in the character of these antibodies. *Acta virol.* **6,** 327—337.

Styk, B., and Hána, L. (1960): Investigations into specific antibodies against A2 influenza viruses and the thermolabile co-factor by means of paper electrophoresis. *Acta virol.* **4,** 365—370, 1960.

Styk, B., and Hána L. (1962): Cofactor and specific antibodies against influenza viruses. V. Interaction of cofactor with antibody and virus studied in adsorption experiments. *Acta virol.* **6,** 508—518.

Styk, B., Hána, L., Franěk, F., Sokol, F., and Menšík J. (1962*a*): Investigations on cofactor and influenza antibodies by density gradient zonal centrifugation. *Acta virol.* **6,** 478.

Styk, B., Menšík, J., Šterzl, J., Hána, L., and Borecký, L. (1962*b*): Cofactor and specific antibodies against influenza viruses. VI. The relationship of cofactor to the so-called natural antibodies as revealed by a study of sera from newborn pigglets. *Acta virol.* **6,** 519—523.

Šterzl, J., Kostka, J., and Lanc, A. (1962): Development of bactericidal properties against gram-negative organism on the serum of young animals. *Folia microbiol.* **7,** 162—174.

Šterzl, J., Kostka, J., Říha, I., and Mandel, L. (1960): Attempts to determine the formation and character of gamma globulin and of natural and immune antibodies in young pigs reared without colostrum. *Folia microbiol.* **5,** 29—45.

Acta virol. **7** : 37—41, 1963

Mumps Virus Infection of HeLa Cells Studied by the Fluorescent Antibody Method

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Received August 22, 1962

Most studies dealing with the assay of mumps virus using fluorescent antibodies have been done in monkeys and chick embryos (Coons *et al.*, 1950; Watson, 1952*a*). Little interest has been paid so far to the relation between the formation of viral antigen and the presence of infectious virus in tissue cultures inoculated with different strains of mumps virus. The present paper reports studies on the multiplication of two mumps virus strains in comparison with the cytological changes occurring in the course of their multiplication in HeLa cells.

Materials and Methods

Virus. Two strains of mumps virus were used: the Enders strain which had undergone 6—8 egg passages in this Institute, and a strain designated 754 which had undergone 14 egg passages in this Institute. The Enders strain was obtained in 1958 from the Ivanovsky Institute of Virology, Moscow and the 754 strain was isolated in 1955 by Dr. K. Žáček, Institute of Epidemiology and Microbiology, Prague. The Enders strain was adapted to the allantois of chick embryo and reached an infectivity titre of $10^{6.5}$ EID_{50}/0.1 ml. and a haemagglutination titre of 1 : 256; the 754 strain was passaged only in the amniotic cavity of chick embryo and reached a haemagglutination titre of 1 : 1,024.

Tissue culture. HeLa cells (serially passaged in a medium containing human serum) were grown on cover glasses in tubes seeded with 100,000 cells in 2 ml. of medium consisting of 89.5 % Hanks' solution, 10 % inactivated horse serum and 0.5 % lactalbumin hydrolysate.

Fluorescent antibody technique. Hyperimmune serum was prepared by treble inoculation of rabbits with infective allantoic fluid mixed with an equal volume of adjuvant (one part lanolin and 3 parts paraffin). The neutralisation titre of the serum thus prepared was 1 : 256. The globulin fraction from the hyperimmune serum was obtained by precipitation with ammonium sulphate of 40 % and 50 % saturation. It was conjugated with fluorescein isothiocyanate at pH 8.8 for 3 hours at 4° C. Excess fluorescein isothiocyanate was removed by adsorption to charcoal. To avoid nonspecific staining, the conjugate was absorbed with acetone-dried mouse liver and chick embryo powder (100 mg. powder per ml. of conjugate).

Cover glasses with grown cell cultures were shortly dried and fixed for 10 minutes in freshly distilled acetone. The cells were stained with the conjugate in a humid chamber for 30 minutes, washed for 30 minutes with physiological saline, pH 7.2, and mounted into a mixture of 10 parts of neutral glycerol with one part phosphate buffered saline, pH 7.2. All these procedures were done at room temperature. Each sample was subjected to three examinations in addition to staining with homologous conjugate, cells from a duplicate culture were stained with heterologous conjugate against Newcastle disease virus. Uninfected cells stained with conjugate against mumps virus served as a further control.

For *cytological examination* the cells were fixed in Bouin's fluid and stained with haematoxylin and eosin.

Titration of virus in the medium from infected HeLa cell cultures was performed in 7—8 days old chick embryos. Haemagglutination titres of the allantoic or amniotic fluids were estimated after 4 days' incubation.

Haemagglutination tests with infected allantoic or amniotic fluids from chick embryos as well as with tissue culture fluids were set up using 0.5 % rooster red blood cells.

Complement fixation reaction (CFR). Tissue cultures inoculated with the Enders or 754 strains were subjected to three cycles of freezing and thawing. The titre of CF antigen was estimated in a box titration using hyperimmune guinea pig serum diluted from 1 : 4 to 1 : 512.

Microscopy and photomicrography. Fluorescent preparations were examined in a C. Zeiss Jena „Grosse Luminiszenzeinrichtung"; in which the carbon arc was substituted by a high pressure mercury vapour lamp HBO 200. U.V. transmitting UG filters, 1, 2 and 4 mm. thick and a GG 9 U.V. absorbing ocular filter were used. Fluorescent photomicrographs were taken on 24 × 36 mm. Agfa Isopan F film with exposures of 1—2 minutes. Pictures of haematoxylin and eosin-stained preparations were taken on Agfa Isochrom 9 × 12 cm. plates.

Results

1. Detection of virus antigen by fluorescent antibodies

As early as 8 hours after inoculation with a large dose ($2 \times 10^{6.5}$ EID_{50}) of the Enders strain, 90% of the HeLa cells contained specifically fluorescing granules, 1—2 μ. in size, irregularly distributed in the cytoplasm. Sixteen—twenty-four hours after inoculation more than 99% of the cells showed antigen in the cytoplasm, appearing either as isolated granules, 1—5 μ. in diameter, or as diffuse fluorescence around the nucleus (Fig. 1). Multinucleated giant cells rich in granular or diffuse antigen were often observed (Fig. 2). The amount of specific antigen in the cells gradually increased until the 3rd day after inoculation, when it filled in many cells a prevailing part of the cytoplasm (Fig. 3). This picture was not substantially changed by the 4th and 5th days after inoculation. Although the distribution and amount of antigen in the majority of cells underwent the course described, individual cells could be found at any stage of infection which, according to the amount and form of antigen deposits reminded of the early stage of infection (Fig. 4).

The appearance of antigen in cells inoculated with only $2 \times 10^{3.5}$ EID_{50} was delayed, the first fluorescence being detected only 48 hours after inoculation. The amount and distribution of antigen at 72—96 hours after inoculation was the same as in cultures inoculated with the large dose of virus.

Similar results were obtained with the chick embryo amnion-adapted 754 strain of mumps virus.

Preparations stained with heterologous conjugate against Newcastle disease virus as well as non-infected cultures stained with the conjugate against mumps virus were negative.

2. Virological examinations

CF antigen could be shown in the medium from HeLa cells 48 hours after inoculation with the Enders strain (Table 1). Neither haemagglutinin nor infectious virus could be demonstrated in either the culture medium or the cell homogenate. Neither infectious virus, nor immunofluorescence could be detected when material from infected cultures was further passaged in HeLa cells.

In contrast to the Enders strain, the 754 strain could be serially passaged in HeLa cells. After 4 passages virus antigen was demonstrated in the cells by immunofluorescence and by CFR; infectious virus was detected by back inoculation into the amniotic cavity of chick embryos.

Table 1. Comparison of the efficacy of different methods for the detection of the Enders strain of mumps virus in HeLa cells

EID_{50} of virus inoculated	Mode of assay	Hours after inoculation						
		8	16	24	48	72	96	120
$2 \times 10^{3.5}$	immunofluorescence	0	0	0	+	+	+	+
	inclusions	0	0	0	0	0	0	0
$2 \times 10^{6.5}$	immunofluorescence	+	+	+	+	+	+	+
	inclusions	0	0	+	+	+	+	+
	EID_{50}	n.t.	n.t.	n.t.	$10^{2.0}$	$10^{1.5}$	n.t.	$<10^{1.0}$
	CF antigen	n.t.	n.t.	n.t.	1 : 2	n.t.	n.t.	1 : 2
	haemagglutination	0	0	0	0	0	0	0

+ = result positive; 0 = result negative; n.t. = not tested

3. Cytological examinations

Twenty-four hours after inoculation with either strain of mumps virus, eosinophilic inclusions appeared in the cytoplasm of HeLa cells. The number of inclusions in cells inoculated with the Enders strain was, however, substantially lower. The quantity of inclusions increased on the second day and reached a maximum 3 days after inoculation (Fig. 5). The further development was characterized by a decrease in the number of inclusions. Giant cells with 7—8 nuclei and numerous inclusions were also seen (Fig. 6). No inclusions or giant cells were observed either in non-inoculated HeLa cells (Fig. 7), or in cells inoculated with $2 \times 10^{3.5}$ EID_{50} of the Enders strain. No cytopathic effect was ever found in cultures inoculated with the Enders or 754 strains.

Discussion

Coons *et al.* (1950) used the fluorescent antibody technique for the assay of mumps virus in the parotis gland of inoculated monkeys. Virus antigen could be detected 4—5 days after inoculation in the secretory and epithelial cells of the gland. The antigen had the shape of fluorescent granules distributed in the cytoplasm of cells. Watson (1952*a*) employed the method of immunofluorescence for studying the development of infection in 8 days old chick embryos inoculated into the amniotic cavity. Virus antigen could be demonstrated 1 and 2 days after inoculation in cells of the amniotic membrane, and 3 days after inoculation in the chorioallantoic membrane, epithelial cells of the skin, in the oropharynx and nasopharynx, whereas after 7 days fluorescence was present in the trachea and bronchi. Virus antigen occurred in the cytoplasm as fluorescent granules of different size.

Watson (1952*b*) demonstrated the presence of antigen in tissue culture cells of various chick embryo tissue. Chick embryo lung cells and human conjunctival cells were employed for the study of mumps virus infection by Traver *et al.* (1960). Virus antigen was demonstrated in the cytoplasm 18 hours after inoculation in form of granules showing intensive fluorescence.

Henle and Deinhardt (1955), Brandt (1958, 1961), and Maas and Mannweiler (1960) observed eosinophilic inclusions in the cytoplasm of cells inoculated with mumps virus. According to Henle *et al.* (1954) a strain of mumps virus which had undergone a low number of passages in the amnion of chick embryos caused a cytopathic effect, whereas a strain of virus adapted to the allantois of the chick embryo did not cause any damage to HeLa cells.

Our results obtained in HeLa cells inoculated with mumps virus are in good accordance with the data of other authors, as concerns the early appearance of antigen. Specific fluorescence could be demonstrated even upon inoculation of HeLa cells with the allantois-adapted Enders strain of mumps virus which did not induce the formation of infectious virus or haemagglutinin in this type of culture. On the other hand, the 754 strain of mumps virus which had been passaged in the amniotic cavity of the chick embryo induced the formation of infectious virus and could be serially passaged in HeLa cells.

Eosinophilic inclusions were demonstrated in HeLa cells inoculated with a large dose of the virus, but not upon inoculation with a lower dose still leading to the appearance of immunofluorescence. Thus there seems to be no relationship between the appearance and amount of antigen and the occurrence of inclusions in inoculated cells.

Summary

The Enders strain of mumps virus, adapted to the allantois of chick embryo does not multiply in HeLa cells. Another strain, designated 754, hitherto passaged only in the amniotic cavity of the chick embryo multiplies and can be serially passaged in HeLa cells.

Specific fluorescence could be detected in the cytoplasm early after inoculation of HeLa cells with either strain of mumps virus.

Eosinophilic inclusions could be demonstrated in the cytoplasm of HeLa cells inoculated with the 754 strain or with a large dose of the Enders strain. The occurrence of inclusions is not connected with the appearance and amount of antigen in inoculated cells.

Acknowledgement. Thanks are due to Mrs. D. Kočišková of this Institute for carrying out the serological tests.

References

Brandt, C. D. (1958): Inclusion body formation with Newcastle disease and mumps viruses in cultures of chick embryo cells. *Virology* **5**, 177.

Brandt, C. D. (1961): Cytopathic action of myxoviruses on cultivated mammalian cells. *Virology* **14**, 1.

Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S. (1950): Localization of antigen in tissue cells. IV. Antigens of rickettsiae and mumps virus. *J. exp. Med.* **91**, 31.

Henle, G., and Deinhardt, F. (1955): Propagation and primary isolation of mumps virus in tissue culture. *Proc. Soc. exp. Biol. (N.Y.)* **89**, 556.

Henle, G., Deinhardt, F., and Girardi, A. (1954): Cytolytic effects of mumps virus in tissue cultures of epithelial cells. *Proc. Soc. exp. Biol. (N.Y.)* **87**, 386.

Mass, G., and Mannweiler, K. (1960): Cytologische und biologische Untersuchungen über das Verhalten des Mumps-Virus in Affennierenepithelkulturen. *Arch. ges. Virusforsch.* **10**, 195.

Traver, M. I., Northrop, R. L., and Walker, D. L. (1960): Site of intracellular antigen production by myxoviruses. *Proc. Soc. exp. Biol. (N.Y.)* **104**, 268.

Watson, B. K. (1952*a*): Fate of mumps virus in the embryonated egg as determined by specific staining with fluorescein-labelled immune serum. *J. exp. Med.* **96**, 653.

Watson, B. K. (1952*b*): Distribution of mumps virus in tissue cultures as determined by fluorescein-labelled antiserum. *Proc. Soc. exp. Biol. (N.Y.)* **79**, 222.

Explanation of Photomicrographs

Fig. 1. HeLa cells 16—24 hours after inoculation with mumps virus, strain Enders. Numerous fluorescing granules or diffuse perinuclear fluorescence in the cytoplasm (× 400).

Fig. 2. HeLa cells 16—24 hours after inocualtion with mumps virus, strain Enders. Multinucleated giant cell showing numerous fluorescing granules and diffuese fluorescence in the cytoplasm (× 400).

Fig. 3. HeLa cells 2—3 days after inoculation with mumps virus, strain Enders. Specific fluorescence in the prevailing part of the cytoplasm (× 400).

Fig. 4. HeLa cells 4—5 days after inoculation with mumps virus, strain Enders. Cells showing diffuse fluorescence along with cells containing a small amount of fluorescing granules (× 200).

Fig. 5. HeLa cells 3 days after inoculation of mumps virus, strain 754. Great number of cytoplasmic inclusions. (Haematoxylin-eosin, × 800).

Fig. 6. HeLa cells 2 days after inoculation of mumps virus, strain 754. Multinucleated giant cell showing a great number of cytoplasmic inclusions. (Haematoxylin-eosin, × 800.)

Fig. 7. HeLa cells, non-inoculated control. (Haematoxylin-eosin, × 800.)

Acta virol. **7** : 42—47 1963

Cytochemical Studies on Nucleic Acids in Cells from Tissue Cultures Infected with Type 5 Adenovirus

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Received August 7, 1962

Nucleic acids and metabolic processes in infected cells have been studied using model infections with adenoviruses. It is known that adenoviruses contain deoxyribonucleic acid (DNA) and that adenoviral inclusions give a positive Feulgen reaction. According to Boyer and Leuchtenberger (1957) and Boyer *et al.* (1959) DNA appears in the inclusions at given stages of their development, at early intervals the Feulgen reaction being negative. Other authors reported only that adenoviral inclusions are Feulgen-positive without indicating the time of their appearance and the dynamics of their development (Barski, 1956; Barski and Cornefert, 1958; Bloch *et al.*, 1957).

It was therefore interesting to investigate the nucleic acid composition of intranuclear inclusions in the course of the development of adenovirus infection using very short intervals of assay with cytochemical methods.

Materials and Methods

Type 5 Adenovirus, strain Rowe, was used. Monolayer cultures of HeLa and SOC stable cell lines on strips of cover glasses were inoculated 3—4 days after seeding with 1000 TCD_{50} of virus. Starting from 6 hours after inoculation, the cultures were examined until complete degeneration (72—120 hours) with 1-hour intervals during the first day and then with 1-day intervals.

To demonstrate the nucleic acids, the cultures were fixed for 15—60 minutes in Carnoy's fluid and stanined according to the classical method of Feulgen for DNA, and with pyronine — methyl green for ribonucleic acid (RNA). In parallel morphological studies the cultures were fixed in Dubosque-Brasil-Bouin mixture for at least 20 minutes and then stained with Orange G — eosin — methylene blue (a modification of the method of Unna — Klisenko, 1961). For fluorescence microscopy, the cultures were stained with acridine orange following fixation in either of these mixtures: 1) 96% ethanol — 125 ml., formalin — 65 ml. and acetic acid — 15 ml., or 2) ethanol — 19 parts and acetic acid — 1 part. The fixed preparations were stained for 30 minutes with a 1 : : 5000 aqueous solution of acridine orange, after which they were passed through a series of increasing concentrations of ethanol and xylene, and mounted in polysterol.

In studying the specificity of nucleic acids, the infected cultures were stained after incubation for 90 minutes at 37° C in a 0.1% aqueous solution of deoxyribonuclease with Mg ions added, or for 90—120 minutes in a 1 mg./ml. solution of ribonuclease.

Uninfected control cultures were examined in parallel, using the same mode of treatment and the same intervals of assay.

The regularity of the dynamics of adenovirus infection was checked by repeated experiments.

To investigate the accumulation of virus in the infected cultures, at intervals of 8, 9, 10, 11, 12, 14, 17 and 24 hours after inoculation they were subjected to 3 brief cycles of freezing and thawing, centrifuged for 10 minutes at 1000 rev./min., and the virus in the supernatant fluid was titrated by routine methods.

Results

In HeLa cell cultures infected with the stock strain of type 5 adenovirus no changes as compared with control cultures were recorded 6 hours after inoculation, i.e. at the first interval of examination.

Preparations stained by the modified method of Unna revealed cells of a typical polygonal shape with numerous protrusions, forming a continuous sheet. The cytoplasm appeared pale violet, the nucleoplasm pinkish-lilac and the nucleoli blue. Most of the cells contained in their cytoplasm perinuclear oxyphilic zones. The rounded nuclei were situated excentrically and each contained 2—4 nucleoli.

Staining according to Feulgen clearly revealed the nuclear structure. The nuclear membrane and the chromatin network stained violet-lilac and the nucleoli appeared as empty places surrounded by a halo of chromatin connected with them. The cytoplasm remained unstained.

In preparations stained with pyronine — methyl green the cytoplasm appeared bright red, the colour being more intensive at one pole of the nucleus and less on cell periphery. The nucleoli were also red. The nuclei stained greenish or pale blue.

Fluorescence microscopy of preparations stained with acridine orange revealed a yellow-green or yellow-orange fluorescence of the cytoplasm and nucleoli. In the nucleus, a green fluorescence was displayed by the membrane and granules of chromatin.

In the majority of experiments first changes appeared in the nuclei of infected cells 8 hours after inoculation in the form of oxyphilic formations surrounded by paler zone (stained by the modified method of Unna), giving a positive Feulgen reaction, and staining greenish-blue with pyronine — methyl green. No changes were found by fluorescence microscopy.

At 9—11 hours the preparations showed the same picture, but staining with acridine orange revealed in the nuclei small dimly outlined formations showing a green fluorescence and numbering 4—5 per nucleus. The nucleoli increased in size and their fluorescence was more intensive than before.

At later intervals, 12—14 hours after inoculation, the inclusions had the form of small rounded formations either with a dense membrane and a faintly staining centre or appearing nearly homogeneous (Figs 1 and 2). In both cases they gave a positive test for DNA (Fig. 3).

At 17—24 hours after inoculation the inclusions remained Feulgen-positive, increased in size and appeared almost in every ocell. Most often they represented large voluminous masses, occasionally showing a honeycomb-like structure. After staining with pyronine — methyl green, blue "cells" or interwoven strands could be seen in them on a pyroninophilic background. Less often small-sized rounded formations with a pale central zone were encountered. Fluorescence microscopy revealed the inclusions as small intranuclear formations displaying a green fluorescence. Oxyphilic inclusions of similar form and structure were seen in preparations made at the same intervals and stained by the modified method of Unna.

At 30—48 hours after inoculation, deformation of the nuclei occurred. Intranuclear inclusions were found in all cells; they were located in the centre of the nuclei as a compact mass. Their form and size were the same whether stained according to Unna of for the nucleic acids. In all experiments the inclusions gave a positive reaction for DNA and a negative one for RNA (Fig. 4). The nucleoplasm of nuclei containing the inclusions did not stain either by

routine methods or by those for nucleic acids. Fluorescence microscopy revealed in the preparations large inclusions displaying an intensive green fluorescence. The nucleoplasm showed no fluorescence and appeared almost black. The fluorescence of the nucleoli was yellow or orange.

At late intervals, from 72—120 hours after inoculation, the majority of the cells became detached from the glass and in the remaining cells rather pronounced changes were observed. The nuclei showed marked deformations and the intranuclear inclusions appeared as dense homogeneous central masses giving a positive Feulgen reaction.

In preparations pretreated with deoxyribonuclease and stained according to Feulgen the colour intensity shown by the nuclei decreased, but the inclusions remained clear-cut. In cells treated with deoxyribonuclease and then stained with pyronine — methyl green the nuclei remained colourless, but the shape and colour of the inclusions did not change. On fluorescence microscopy of cells pretreated with deoxyribonuclease and stained with acridine orange, the intensity of the fluorescence of the nuclei decreased somewhat, but the shape of the inclusions and the intensity of their fluorescence remained almost unaltered.

In preparations stained for RNA after the action of ribonuclease (method of Brachet) the cytoplasm and nucleoli remained unstained and the intranuclear inclusions retained their structure and colour. Preparations treated with ribonuclease and then stained according to Feulgen looked like preparations untreated with the enzyme. Fluorescence microscopy of preparations pretreated with ribonuclease revealed changes in the fluorescence of the cytoplasm and nucleoli — the yellow-orange fluorescence was almost absent.

Because treatment with deoxyribonuclease did not appreciably affect staining of the inclusions according to Feulgen, in further experiments infected cells were pretreated with pepsine or veronal buffer pH 9.0 and only then with deoxyribonuclease. The cultures were incubated for 2 hours in a 0.02% pepsine solution or for 90 minutes in veronal buffer pH 9.0 at 37° C and then treated with deoxyribonuclease. As controls, cultures treated only with pepsine or veronal buffer were used. Nor in these experiments did the colour or shape of the intranuclear inclusions change on subsequent staining according to Feulgen (Fig. 5), although there were changes in the external appearance of the cultures. Following pepsine treatment the cell sheet went asunder, the cells became rounded and shrunken, there was destruction of the cell walls (Fig. 6) and fluorescence microscopy revealed a change in the fluorescence of the cytoplasm to a yellowish-copper colour.

As a further control of the specificity of staining for nucleic acids, treatment with 5% trichloroacetic acid, which removes both nucleic acids under appropriate conditions (for 15 minutes at 90° C), was employed. In this case the cultures did not stain according to Feulgen or with pyronine — methyl green and showed only an extraordinarily weak monotonous fluorescence when examined by fluorescence microscopy.

Similar results were obtained in experiments in which SOC cell cultures infected with type 5 adenovirus were studied.

The results of virus titrations from infected cultures at various intervals after inoculation are presented in Table 1. It is evident that first detectable amounts of virus appeared 10 hours after inoculation and corresponded to

Table 1. Dynamics of cytological and cytochemical changes and of virus accumulation in SOC cell cultures infected with type 5 adenovirus

Hours after inoculation	Method of staining				Titre of virus (log TCD_{50} per ml.)
	Unna (modified)	Feulgen	Pyronine-methyl green	Acridine orange	
8	—	—	—	—	—
9	—	—	—	—	—
10	+	+	+	+	1.0
11	+	+	+	+	2.0
12	+	+	+	+	2.0
14	+	+	+	+	2.0
17	++	++	++	++	3.0
24	+++	+++	+++	+++	> 3.0

— absence of inclusions or no virus detected; + numbers of small inclusions; ++medium sized inclusions in each sight-field; +++ large polymorphous inclusions in the majority of cells.

10 TCD_{50}/ml. At this interval small oxyphilic, Feulgen-positive formations were observed in the cell nuclei. The virus titre increased further on, reaching a maximum 24 hours after inoculation, when intranuclear inclusions were present in the majority of the cells.

Discussion

The investigations reported above showed that type 5 adenovirus infection of HeLa and SOC cell cultures is characterized by cellular changes accompanied by the formation of intranuclear inclusions. First changes in the cell nuclei appeared 8—10 hours after inoculation as oxyphilic formations giving a positive Feulgen reaction. Later on the inclusions increased in size, acquired a complex structure and contained DNA for the whole period, treatment of the cultures with deoxyribonuclease causing no changes in their staining propertiees.

A comparison of the results of complex cytochemical investigations on the composition of inclusion caused by type 5 adenovirus shows that, as distinct from the reports by Boyer and Leuchtenberger (1957) and Boyer *et al.* (1959), DNA was present in the inclusions during the whole process of infection.

It is interesting to note that in a recent publication Ginsberg and Dixon (1961) reported on the demonstration by biochemical methods and isotope techniques of a rapid increase in the synthesis of saline soluble DNA fraction in adenovirus-infected cultures preceding the appearance of infective virus particles. According to our opinion these results concur with those of the present investigations.

Reports by Armstrong and Hopper (1959) on the effect of pepsine on staining properties of the inclusions and by Epstein and Holt (1960) on a similar effect of veronal buffer pH 9.0 were not confirmed in our experiments.

It must also be pointed out that in the present investigations there was a close parallelism between the appearance of biologically active virus in the cultures inoculated and that of intranuclear inclusions.

Summary

1. First intranuclear inclusions caused by type 5 adenovirus in HeLa and SOC cell cultures appeared 8—10 hours after inoculation as small oxyphilic, DNA-containing formations 1—2 μ in diameter.

2. Later on the inclusions increased in size, acquired a complex structure and filled the whole nucleus. At late stages of infection the inclusions have shrunken and appeared as homogeneous oxyphilic masses situated in the centre of the nucleus and giving a positive Feulgen reaction. Stained with acridine orange, the inclusions displayed bright green fluorescence.

3. Treatment of infected cultures with nucleases, or with pepsine or veronal buffer pH 9.0 and subsequently with deoxyribonuclease did not affect the structure and staining properties of the intranuclear inclusions.

4. Biologically active virus appeared in the infected cultures simultaneously with intranuclear inclusions and the increase in the virus titre paralleled the dynamics of inclusion development.

5. Intranuclear inclusions in cells from cultures infected with type 5 adenovirus contained DNA for the whole period of infection, from the moment of their appearance until complete degeneration of the cultures.

References

Armstrong, J. A., and Hopper, P. K. (1959): Fluorescence and phase contrast microscopy of human cell cultures infected with adenovirus. *Exp. Cell Res.* **16,** 584.

Barski, G. (1956): Caractère spécifique de la lésion cellulaire causée *in vitro* par les virus du groupe APC et sa valeur diagnostique. *Ann. Inst. Pasteur* **91,** 614.

Barski, G., and Cornefert, F. (1958): Aspects distinctes des lésions cellulaire causées *in vitro* par différents types d'adenovirus. *Ann. Inst. Pasteur* **94,** 724.

Bloch, D. P., Morgan, C., Godman, G. C., and Howe, C. (1957): A correlated histochemical and electron microscopic study of the intranuclear crystalline aggregates of adenovirus (Ri-APC virus) in HeLa cells. *J. biophys. biochem. Cytol.* **3,** 1.

Boyer, G. S., and Leuchtenberger, C. (1957): Cytological and cytochemical studies of HeLa cells infected with adenoviruses. *J. exp. Med.* **105,** 195.

Boyer, G. S., Denny, F. W., and Ginsberg, H. S. (1959): Sequential cellular changes produced by types 5 and 7 adenovirus in HeLa cells and human amniotic cells. *J. exp. Med.* **110,** 827.

Epstein, M. A., and Holt, S. J. (1960): Site and nature of adenovirus nucleic acid. *Nature (Lond.)* **187,** 1050.

Ginsberg, H. S., and Dixon, M. K. (1961): Nucleic acid synthesis in HeLa cells infected with types 4—5 adenovirus. *J. exp. Med.* **113,** 283.

Klisenko, G. A. (1961): Staining of viral inclusions in fixed tissue culture preparations. *Vop. Virusol.* **6,** 499 (in Russian).

Explanation of Photomicrographs

Fig. 1. HeLa cell culture 14 hours after inoculation with type 5 adenovirus. Small rounded inlusions 1—2 μ in diameter surrounded by a narrow paler zone can be seen. Stained by the modified method of Unna. × 1200.

Fig. 2. The same culture stained with pyronine — methyl green. × 1200.

Fig. 3. The same culture stained according to Feulgen. Rounded intranuclear Feulgen-positive inclusions indicated by arrows. × 1200.

Fig. 4. HeLa cell culture 48 hours after inoculation with type 5 adenovirus. Large Feulgen-positive inclusions situated as compact masses in the centre of the nuclei. Stained according to Feulgen. × 1200.

Fig. 5. The same culture stained according to Feulgen after pretreatment with pepsine and deoxyribonuclease. The shrunken nuclei stained very weakly. The intranuclear inclusions of the same appearance as in Fig. × 1200.

Fig. 6. The same culture stained with pyronine — methyl green after pretreatment with pepsine. The cell membranes and cytoplasm destroyed, the cell contours dim. The inclusions retained their structure and shape. × 1200.

Acta virol. 7 : 48—53, 1963

Morphological and Cytochemical Study of HEp-2 Cell Cultures Persistently Infected with Tick-borne Encephalitis Virus

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Received June 6, 1962

Persistent infection of stable cell lines with tick-borne encephalitis virus has been described by Andzhaparidze and Bogomolova (1961) and Mayer (1962). This form of infection is characterised by a long-lasting virus carrier state without apparent degeneration of cells. It can be supposed, however, that the long-term virus carrier state has to bring about changes in different properties of the infected cells. The aim of the present investigation was the study by morphological and histochemical methods of cell lines persistently infected with tick-borne encephalitis virus.

Materials and Methods

The major part of the investigations was carried out in HEp-2 cell cultures inoculated with a single dose of the Sofin strain of tick-borne encephalitis virus. Some experiments, which yielded analogous results, were done with cynomolgus monkey heart and HeLa cell lines. Strains Pan and Ix-10 of tick-borne encephalitis virus and the Absettarov strain of bi-phasic meningoencephalitis virus were also used for inoculating HEp-2 cells.

Persistently infected cells were grown in medium 199 with 10% bovine serum and subcultured every 7—10 days.

Before subjected to morphological and histochemical studies, the persistently infected cells were grown on mica sheets in tubes. For morphological studies, the cells were fixed in Bouin's fluid and stained with haematoxylin and eosin. Histochemical studies included reaction for ribonucleic acid, glycogen and the activities of succinic dehydrogenase and cytochrome oxidase. The methods of histochemical investigations have been described previously (Zalkind *et al.*, 1962).

Results

Morphology of HEp-2 cells persistently infected with tick-borne encephalitis virus

HEp-2 cells persistently infected following single inoculation with tick-borne encephalitis virus were observed for 24 months (61 passages). Morphological differences between infected and control cultures were noticed at early passage levels of persistently infected cells. A marked heterogeneity of the cell population was found in the 12th passage: in addition to stellate cells, typical of control cultures, small round cells, as well as uninuclear and multinuclear giant cells were observed which were absent from, or occurred only very rarely in control cultures. The appearance of giant cells apparently represents the first morphological sign of a persistent infection of stable cell lines. Otherwise the character and intensity of growth of infected and control cultures were the same. A striking change in the morphology of persistently infected cells occurred between the 14th and 29th passages. Instead of dense conglomerates of polygonal cells, the culture in the 29th passage was characterised by small

round cells appearing either isolated or in small groups. This picture remained without major changes during the whole time of cultivation of persistently infected cells (61 passages). The most characteristic difference between the infected and control cultures was the lower rate of multiplication of the former. At the same interval after seeding, when a confluent layer of mostly polygonal cells was formed in control cultures, only isolated round or stellate cells, or small groups of them were present in infected cultures (Fig. 1a, b). These differences were well marked after 2—3 days of cultivation; at later intervals they were usually obliterated. The changes described were the most marked in cells persistently infected with the Sofin, Pan and Absettarov strains, whereas HEp-2 cells carrying the Ix-10 strain showed less marked and less stable reduction of the growth rate.

Histochemical studies of HEp-2 cells persistently infected with tick-borne encephalitis virus

With respect to the observed lower multiplication rate of persistently infected cultures it was of interest to show what differences in the metabolism of these cultures could be demonstrated by histochemical methods.

Ribonucleic acid (RNA). An increased RNA content of infected cells was observed, which was manifested as follows: 1) Infected cultures contained round and stellate cells usually rich in RNA deposits in greater numbers than control cultures; and 2) in some experimental series there was an increase in RNA content also of polygonal cells occurring more frequently in uninfected than in persistently infected cultures. Thus the higher RNA content of persistently infected cultures was connected with an increase both in the number of "young" cells rich in RNA, and in the RNA content of "older" cells, usually containing only little RNA. The RNA deposits in both infected and control cultures were homogeneous and consisted of small granules. Only rarely large inclusions of RNA were seen in infected cells. Differences in the quantity of deposited RNA were marked especially at early intervals of cultivation (2—3 days) and were obliterated after 7—10 days. No differences in the dynamics of RNA deposition could be observed between the different virus strains.

Glycogen. An increase in the glycogen content of infected cells was observed in the great majority of experiments (Fig. 2a, b). The stimulation of glycogen accumulation manifested itself: 1) by an increase in the number of cells showing considerable deposits of this substance; 2) by an increase in the size and intensity of staining of the inclusions; and 3) in a change of the character of the inclusions. In cells of control cultures glycogen deposits were found almost exclusively in the form of small granules. Numerous granular inclusions, filling up the whole cytoplasm were most frequently found in infected cells. Klasmatosis was sometimes observed, leading to the liberation of a part of the glycogen into the surrounding medium. Amorphous drop-like deposits of lyoglycogen, reaching occasionally large dimensions, could be found in some cells. The increased glycogen content of infected cells was preserved for 7—10 days, i.e. for the whole observation period.

No strain-specific peculiarities in the character of glycogen accumulation

could be noticed in cells infected with different virus strains. However, in cells persistently infected with the Ix-10 strain heavy granular inclusions of glycogen were more frequent than in cells infected with the other strains.

Oxidative enzymes. At present, much attention is being paid to oxidative enzymes, particularly to succinic dehydrogenase and cytochrome oxidase. These enzymes are of great importance in the cell metabolism since they are topographically bound with mitochondria. Thus it is very desirable to investigate the activities of succinic dehydrogenase and cytochrome oxidase in cells cultivated *in vitro*, showing intensive metabolic activities, particularly of oxidative processes.

Basing on these considerations we studied the activities of succinic dehydrogenase and cytochrome oxidase in cultures of HEp-2 cells persistently infected with tick-borne encephalitis virus. The level of enzymatic activity was determined according to 1) the number of cells showing positive reaction, and 2) the size, number and intensity of diformasan deposits in cells.

An increased activity of succinic dehydrogenase in infected cells as compared with control cells was observed in the great majority of experiments during the whole period of observation. This increased activity manifested itself both in an increased number of cells showing diformasan deposits and in the different character of the deposits, which were crystalline in infected cells, whereas small and medium-sized granules prevailed in cells of control cultures (Fig. 3a, b).

Studies on the activity of cytochrome oxidase yielded different results. A considerable decrease in the activity of this enzyme was noticed in infected cells, the difference from control cells being up to 2—3 conventional units. The differences were based on the number of cells showing positive reaction and on the intensity of the precipitate; no differences could be seen in the form of diformasan deposits in infected and control cultures (Fig. 4a, b).

Cultures persistently infected with the Ix-10 strain did not show significant changes in enzyme activity.

Discussion

The data obtained make it possible to conclude that HEp-2 cells persistently infected with tick-borne encephalitis virus markedly differ in some features from cells of control cultures.

The differences are first of all morphological, characterised mainly by the prevalence of small rounded cells forming peculiar conglomerates. It can be assumed that these differences are the result of the somewhat slower rate of multiplication of infected cells leading at early stages of cultivation to the prevalence of morphologically "juvenile" cells as compared with control cultures. Andzhaparidze and Bogomolova (1961) also noticed a slower growth rate of cultures infected with tick-borne encephalitis virus. The delay in the multiplication of cells was temporary and at the end of cultivation the infected cultures usually did not differ morphologically from control cultures. The latter fact indicates that the persistent infection does not cause any irreversible damage to the cells, but only retards the development of the culture, affecting

in a particular way the metabolism of cells. The change in the metabolism of infected cells was shown by histochemical examinations which revealed an increased RNA and glycogen accumulation in infected cells. It remains obscure, however, whether the increased accumulation of RNA is actually caused by a stimulation of the nucleoprotein metabolism. It is possible that the increased RNA accumulation in persistently infected cultures is due to the fact that, with respect to the slower rate of multiplication, young cellular forms prevail, which are known to be rich in RNA. Nor can, however, another explanation be excluded; namely that a stimulation of the nucleoprotein metabolism actually could take place in persistently infected cells connected with the presence of virus in the cell and resulting in the intensification of synthetic processes in the infected cell.

An increase in the glycogen content was noticed during the whole time of cultivation, including late intervals at which the delay in the growth rate of infected cultures became obliterated. This supports the assumption that an actual change in the carbohydrate metabolism of the cell took place in the cultures studied, not connected with the delay in the growth of the culture. It is not clear, however, whether there occurs a stimulation of this metabolism. The appearance of lyoglycogen and its large deposits in the cells apparently has to be considered as a loss of the ability of the cell to bind glycogen with structural elements of the cytoplasm at the same time when the ability to its deposition is preserved. It must be mentioned that according to Zalkind and Izakova (1962) the appearance of lyoglycogen can be observed in cultured cells inoculated with poliovirus and that large glycogen deposits were found by Izakova (1962) in senescent cells of stable lines following the supply of excess nutritive substances when changing the medium.

The observed increase in the activity of succinic dehydrogenase in infected cultures agrees well with the numerous reports on the change of enzyme activity in virus-infected culture cells. Matzelt *et al.* (1958) observed that the activity of glycolytic enzymes increased in kidney cell cultures inoculated with poliovirus. Using the same system, Kovacs (1956) was able to show that poliovirus affects the activity of alkaline phosphatase, causing its decrease before the appearance of cytopathic effect and thereafter some stimulation. Zaslavsky and Amchenkova (1961) observed an intense decrease of the activity of succinic dehydrogenase in HEp-2 cells infected with poliovirus. The decrease in the activity preceded the appearance of the cytopathic effect. Multiplication of tick-borne encephalitis virus occurs in persistently infected HEp-2 cells (Andzhaparidze and Bogomolova, 1961). Naturally, the multiplication of virus is accompanied by a stimulation of different functions of the cell, including oxidative processes. The intensification of oxidative processes in infected cells is reflected in the increased activity of succinic dehydrogenase.

The reason of the observed decrease in the activity of cytochrome oxidase is questionable. A consistent interpretation of these data is hardly possible, but it can be shown that according to Zaslavsky (1961) there is a certain antagonism between the activities of the above mentioned two enzymes in stable cell lines. For example at early stages of cultivation a high activity of

succinic dehydrogenase and a low activity of cytochrome oxidase are found at the same time. It is possible that a competition exists between the two enzymes in the transfer of electrons in the oxidative chain. It can be assumed that the viral infection stimulates those links of the oxidative cycle which are connected with the activity of succinic dehydrogenase. As a result of the existing antagonism between the activities of the two enzymes, this leads to the simultaneous inhibition of the activity of cytochrome oxidase.

Thus, HEp-2 cells, persistently infected with tick-borne encephalitis virus, show changed physiological properties despite the lack of a cytopathic effect. The effect of virus manifests itself in a slower rate of growth of the culture and in a change of some metabolic activites. The peculiar morphology of infected cultures reflects the changed functions of the persistently infected cells.

It must be mentioned that the results obtained with the Ix-10 strain of tick-borne encephalitis virus were less marked than, and differed from, those obtained with the other strains. Apparently the persistent infection of HEp-2 cells with this strain offers some peculiarities which are worth of a separate study.

Summary

1. Persistent infection of HEp-2 cell cultures with tick-borne encephalitis virus leads to morphological changes which are manifested by the appearance of conglomerates of small rounded cells and by a general delay in the growth rate of infected as compared with control cells. The persistent infection does not lead to the appearance of cytopathic changes.

2. An increase in the accumulation of ribonucleic acid and in the activity of succinic dehydrogenase of persistently infected cells was shown by histochemical methods. These resuls indicate that there is a stimulation of nucleoprotein metabolism and of oxidative processes in infected cells.

3. The increased accumulation of glycogen is accompanied by the appearance of considerable amounts of lyoglycogen. This indicates that the persistently infected cell is unable to metabolize carbohydrates which had accumulated in it.

References

Andzhaparidze, O. G., and Bogomolova, N. N. (1961): Interaction between tick-borne encephalitis virus and sensitive cells *in vitro*. II. Latent infection of cells. *Vop. Virusol.* **7**, 343 (in Russian).

Izakova, L. P. (1962): Dynamics of glycogen in cells cultivated *in vitro*. *Citologiya*, **4**. 427 (in Russian).

Kovacs, E. (1956): Comparative biochemical studies on normal and on poliomyelitis virus-infected tissue cultures. Profound alteration of acid and alkaline phosphatase activity in infected rhesus kidney cells. *J. exp. Med.* **104**, 589.

Matzelt, D., Homann, J., and Lennartz, H. (1958): Das Verhalten glykolytischer Enzymaktivitäten in Gewebekulturen vor und nach Beimpfung mit Virus. II. Messungen and Gewebekulturen, die mit Poliomyelitisvirus, Typ I, infiziert wurden, im Vergleich zu normalen Gewebekulturen. *Biochem. Z.* **330**, 260.

Mayer, V. (1962): "Partial" cytopathic effect of tick-borne encephalitis virus — a consequence of persistent infection of stable cell lines. *Acta virol.* **6**, 92.

Zalkind, S. Ya., Andzhaparidze, O. G., Bogomolova, N. N., and Fokina, A. M. (1962): Morphological and cytochemical investigations on the effects of tick-borne encephalitis virus on cells from tissue cultures. *Acta virol.* **6**, 447.

Zalkind, S. Ya., and Izakova, L. P. (1962): Dynamics of glycogen in non-infected and virus-

infected cells cultivated *in vitro*. Proc. 2nd Conf. on Chemistry and Metabolism of Carbo hydrates (in Russian).

Zaslavsky, V. G. (1961): Cytochemical determination of the activity of some enzymes in primary cultures and stable cell lines. *Virusnye infektsii i protivovirusnye preparaty* (Viral infections and antiviral preparations) **2**, 316 (in Russian).

Zaslavsky, V. G., and Amchenkova, A. M. (1961): Cytochemical study of some enzymes and protein thiol-groups in tissue culture cells infected with poliovirus. *Virusnye infektsii i protivovirusnye preparaty* (Viral infections and antiviral preparations) **2**, 323 (in Russian).

Explanation of Photomicrographs

Fig. 1. a — HEp-2 cells persistently infected with tick-borne encephalitis virus; 55th passage, 5 days after seeding.
b — Non-infected culture of HEp-2 cells.
Fixed in Bouin's fluid, stained with haematoxylin and eosin. × 500.

Fig. 2. a — Glycogen deposits in HEp-2 cells persistently infected with tick-borne encephalitis virus; 52nd passage, 7 days after seeding. Deposits of lyoglycogen can be seen.
b — Non-infected culture of HEp-2 cells.
Fixed and stained according to Shabadash. × 500.

Fig. 3. a — Activity of succinic dehydrogenase in HEp-2 cells persistently infected with tick-borne encephalitis virus; 42nd passage, 4 days after seeding.
b — Non-infected culture.
Histochemical method of Seligman and Rutenburg as modified by Hirono. × 500.

Fig. 4. a — Activity of cytochrome oxidase in HEp-2 cells persistently infected with tick-borne encephalitis virus 42nd passage. 4 days after seeding.
b — Non-infected culture.
G-Nadi-oxidase reaction according to Moog (*J. cell. comp. Physiol.* **22**:223, 1944) × 500.

Acta virol. 7 : 54—60, 1963

Methods of Preparation and Immunogenic Properties of a Killed Tissue Culture Vaccine against Tick-borne Encephalitis

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Received September 8, 1962

The 20 years' experience of the study of tick-borne encephalitis in the U.S.S.R. demonstrated the high efficacy of the formolized mouse brain vaccine developed in 1938—1940 by Smorodintsev *et al.* (1940, 1941).

If, however, the virus-infected mouse brain got contaminated with bacteria before formalization, occasional cases of allergic encephalitides occurred in men inoculated with such mouse brain vaccine. To avoid this hazard, a strict control has to be ensured of the bacterial sterility of the brain tissue used for the preparation of vaccine. Another way of avoiding the danger of allergic encephalitides is to exclude completely the use of mouse brain by other sources of large amounts of virus. The most promising in this respect appears to be the use of monolayer cultures of chick embryo cells. High yields of virus obtained in this type of culture served as source for the preparation of immunogenic killed formolized vaccines (Daneš and Benda, 1960*a*, *b*; Ilyenko, 1960; Levkovich and Zasukhina, 1960*a*, *b*; Spitsina, 1961).

The tissue culture used for the propagation of virus for a vaccine 1) has to provide regular yields of tick-borne encephalitis virus as high as 3×10^7 to $10^{7.5}$ LD_{50} per ml., using a maintenance medium containing minimal quantities of protein, and 2) must not contain spontaneous viruses of developing chick embryos, e.g. Newcastle disease or avian leukosis viruses.

The aim of the present experiments was to investigate the conditions of the preparation of a killed vaccine against bi-phasic and tick-borne encephalitides from tissue culture, namely the selection of virus strain, study of its multiplication in culture, and the determination of optimal formalin concentration, and temperature and duration of formalization ensuring the inactivation of virus along with a maximal preservation of its immunogenic properties.

Materials and Methods

Virus. If not stated otherwise, the Absettarov strain of bi-phasic meningoencephalitis virus which had undergone 16—18 mouse passages since its isolation, was used in all experiments.

Tissue culture. Monolayer cultures of trypsinized chick embryo cells were grown in medium consisting of Hanks' solution with 2% calf serum inactivated for 30 minutes at 56° C.

Propagation of virus and its inactivation with formalin. Cultures were inoculated with virus from homologous cells, using 10^3 mouse LD_{50} per 1 ml. of medium, and incubated at 37° C for 72 hours. The culture fluid was filtered through cotton-wool. Formalin was then added to a final concentration of 1 : 500, 1 : 1,000, 1 : 2,000 or 1 : 4,000, and the mixtures were incubated at 37°, 20° or 4° C for different periods of time.

The presence of live virus in formolized vaccine was checked by intracerebral inoculation of white mice with 0.03 ml. of the material examined, as well as by inoculating chick embryo cells with 15—20 ml. of the same material. Virus multiplication in tissue cultures was checked by intracerebral inoculation of white mice with the culture fluids.

The immunogenic properties of the vaccine were examined by estimating the resistance of immunised white mice to intraperitoneal inoculation.

Results and Discussion

Various strains of bi-phasic and tick-borne encephalitis viruses were tested in order to select a virus strain giving the highest yield of virus in chick embryo cells. In view of the considerable differences observed in the intensity of multiplication of the 36 strains examined (Table 1), which reached two log units when

Table 1. Yields of different strains of bi-phasic meningoencephalitis and tick-borne encephalitis viruses in chick embryo cell cultures

Viruses	Number of strains tested	Number of strains yielding log i.c. mouse LD_{50}/0.03 ml. culture fluid			
		6.5—7.0	6.0—6.5	5.5—6.0	5.5—5.0
Bi-phasic meningo-encephalitis	20	1	8	5	6
Tick-borne encephalitis	15	—	8	7	—
Louping-ill	1	—	—	1	—

expressed in LD_{50} values, all principal studies were performed with the strain Absettarov of bi-phasic meningoencephalitis virus less virulent for man than the other viruses and gives regular high yields in tissue cutlture. Attempts at adapting different virus strains to tissue culture by repeated subpassages did not lead to an increase in their yield (Table 2).

In pilot tests we investigated the rate of inactivation of bi-phasic meningoencephalitis virus in tissue culture fluid at various concentrations of formalin and at different temperature (Fig. 1). Virus inactivation at 37° C took place within 1—4 days, depending on the concentration of formalin.

Experience obtained in preparing the mouse brain vaccine against tick-borne encephalitis showed that the inactivation by formalin at 37° C destroyed

Table 2. Yields of bi-phasic meningoencephalitis and louping-ill viruses after 1—100 passages in chick embryo cell cultures (log i.c. mouse LD_{50}/0.03 ml.)

Virus strain	Number of passages									
	1	10	20	30	40	50	60	70	80	100
Absettarov	6.0	5.0	5.5	5.2	6.2	5.2	6.0	5.5	6.5	—
173	6.2	5.5	5.0	5.0	4.2	5.0	6.0	5.0	—	—
20	6.2	5.0	5.5	5.2	4.2	—	—	—	—	—
Louping-ill	5.0	5.7	5.7	4.5	5.5	6.0	6.4	5 5	6.2	5.7

— Not tested

not only the infectivity, but also the immunogenicity of the virus. The inactivation was much delayed at 4° C, requiring 14 days with a formalin concentration of 1 : 1,000, 20—25 days at a concentration 1 : 2,000, or 35—40 days at a concentration of 1 : 4,000. The time required for virus inactivation at 20° C was in between the times necessary for inactivation at 37° and 4° C.

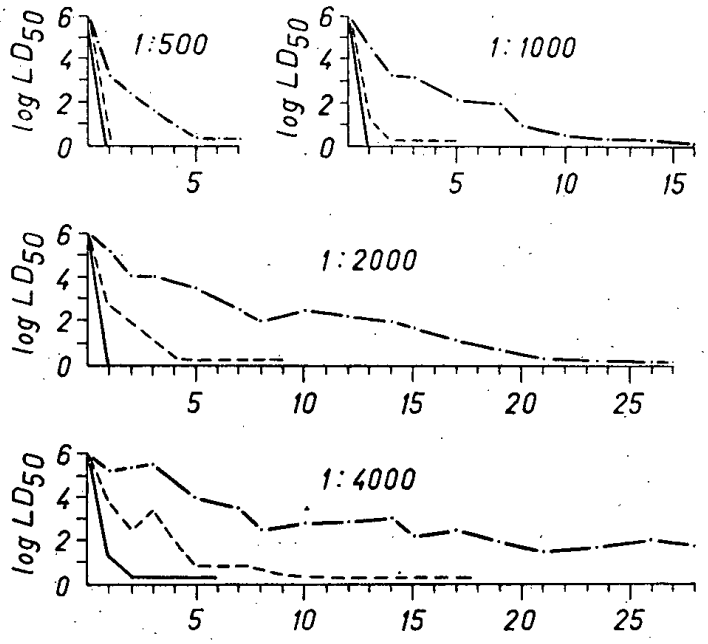

Fig. 1.
Course of inactivation of bi-phasic meningoencephalitis virus in tissue culture vaccine at different temperatures and formalin concentrations 1 : 500, 1 : 1000, 1 : 2000 and 1 : 4000.
Abscissae: time of inactivation in days
Temperature of inactivation: ——— 37° C, — — — — 20 °C, — · — · — 4° C.

Vaccines prepared with various formalin concentrations at different temperatures were administered to white mice, the resistance of which was then assayed by intraperitoneal inoculation of different doses of virus. It was found that a formalin concentration of 1 : 500 fully destroyed the immunogenicity of virus irrespective of the temperature used for inactivation. At an inactivation temperature of 4° C and a formalin dilution of 1 : 1,000, the vaccine exerted a low immunogenicity. At higher temperatures, formalin diluted 1 : 1,000 destroyed the immunogenicity of virus. A regular and well marked immunogenicity was displayed by vaccines prepared at 4° C with formalin diluted 1 : 2,000 or 1 : 4,000 (Table 3).

The study of the dynamics of virus inactivation at various formalin concentrations revealed that the inactivation of virus at a formalin concentration of 1 : 4,000 is rather slow and irregular. Therefore formalin diluted 1 : 2,000 was used for the inactivation of virus in the experiments proper.

Thus we cannot agree with Levkovich and Zasukhina (1960*a*, *b*) who recomended to carry out inactivation of tick-borne encephalitis virus in a tissue

culture vaccine at 37° C. Great experience obtained in preparing killed brain vaccines and the data on the tissue culture vaccine given above show that the immunogenicity of virus is readily destroyed under such conditions.

Table 3. The relationship between immunogenicity of the tissue culture vaccine and the concentration of formalin and temperature used for inactivation

Inactivation temperature	Dilution of formalin			
	1 : 500	1 : 1,000	1 : 2,000	1 : 4,000
37° C	0*	0	0	1.2
20° C	0	0	0	3.7
4° C	2.0	3.7	5.3	5.2

* resistance index of immunised mice (in log LD_{50}; 0 means no resistance).

A full destruction of the vaccine's immunogenicity following virus inactivation at 37° C, was also observed by Semenov *et al.* (1961).

A comparison of parallel safety tests of the vaccine performed in mice (inoculated intracerebrally) and in tissue cultures showed the advantage of the latter, since tissue cultures can be inoculated with 300—600 times greater volumes than white mice.

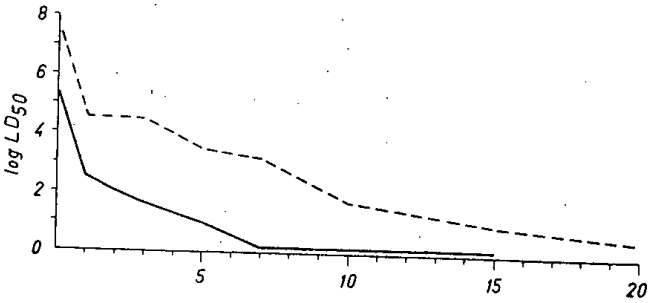

Fig. 2.
Course of inactivation of bi-phasic meningoencephalitis virus in tissue culture vaccine followed by parallel tests done in mice (————, 0.03 ml. intracerebrally) or tissue cultures (— — — —, 1 ml.)
Conditions of inactivation: formalin 1 : 2000, temperature +4° C.
Abscissae: time of inactivation in days

Fig. 2 demonstrates the dynamics of inactivation of bi-phasic meningoencephalitis virus in the course of its interaction with formalin as revealed by tests done in mice or tissue cultures. At late intervals of inactivation, when no virus could be recovered by the inoculation of mice, the presence of virus could be demonstrated in tissue culture.

It is to be noted that it is not desirable to filter the vaccine, not even through filters of a large porosity, e.g. through glass filters, since this markedly reduced the immunogenicity of the preparation. For instance, when a vaccine yielded a resistance index in mice of 5.0 before filtration, the index decreased to 1.6 after filtration.

Virus inactivation in the tissue culture vaccine by U.V. irradiation, using an apparatus described by Piskareva *et al.* (1959), did not warrant an active and safe preparation.

Tests on the use of betapropiolactone for inactivating tick-borne encephalitis virus revealed that this substance diluted 1 : 1,000—1 : 4,000 in-

activated the virus within 24 hours, diluted 1 : 5,000 within 72 hours, and diluted 1 : 10,000 within 25 days. Higher dilutions of betapropiolactone failed to ensure a safe inactivation of virus (Fig. 3). The immunogenic activity of

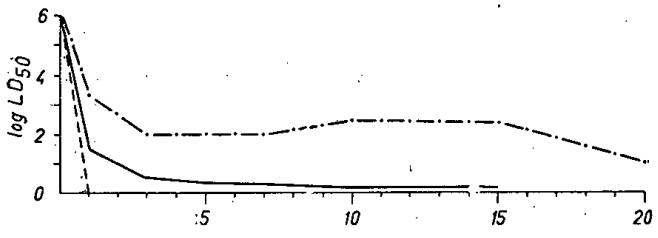

Fig. 3.

Course of inactivation of bi-phasic meningoencephalitis virus in tissue culture vaccine at different concentrations of betapropiolactone. Temperature of inactivation +4° C.
Concentrations of betapropiolactone: ———— 1 : 5000, ———— 1 : 10000, — · — · — 1 : 15000
Abscissae: time of inactivation in days.

vaccines inactivated with betapropiolactone was not less than that of formolized preparations. The resistance indices in mice vaccinated with four different lots of vaccine inactivated with betapropiolactone diluted 1 : 10,000 or 1 : 5,000 varied from 5.0 to 5.5, and the indices of mice vaccinated with 14 different lots of vaccine inactivated with formalin diluted 1 : 2,000 varied from 4.2 to 6.0.

Table 4. Effect of storage at 4° C on the activity of tissue culture vaccine

Vacc. No.	Resistance indices (log LD_{50}) after months of storage							
	1	3	4	5	7	10	13	19
1	4.8	—	—	4.3	—	3.5	—	—
2	4.3	4.5	—	4.5	4.1	—	5.0	—
3	6.6	—	—	—	—	5.3	4.4	2.7
4	5.6	—	—	—	5.3	—	—	—
5	4.5	—	5.1	—	—	5.0	—	—
6	6.2	—	4.4	—	3.7	2.0	—	0.5

The formolized tissue culture vaccines prepared were sufficiently immunogenic and regularly caused immunity of vaccinated mice. The activity of liquid formolized tissue culture vaccines was preserved usually for over 6 to 8 months (Table 4), and a time of storage of 6 months appears warranted for production purposes. The drying of the vaccines considerably prolongs the admissible time of storage.

The reactogenicity and immunogenicity of the tissue culture vaccines prepared were tested in a limited number of humans. Preliminary trials of the vaccine in over 100 persons revealed the complete areactogenicity of the preparation: neither general nor local reactions were registered.

To test the immunogenicity three doses of the tissue culture vaccine of 2.3 and 3 ml., respectively, were administered, the second dose 7—10 days after the first, and the third dose 3 weeks after the second one. Blood samples

were taken before vaccination and one month after the last dose of vaccine, and the paired samples examined for neutralising antibodies in white mice at the same time.

Table 5. Vaccination of men with the killed tissue culture vaccine against tick-borne encephalitis

Dilution of vaccine	Number of persons vaccinated, who had no neutralising antibodies before vaccination	Number of persons who developed neutralising antibodies after vaccination	
		Total	In %
undiluted	60	40	66
1 : 5	14	6	42
1 : 25	12	3	25

A total of 113 persons were vaccinated, but only 86 of them had no neutralising antibodies before vaccination. Of these 60 persons were given the undiluted preparation, the rest were inoculated with vaccine diluted 1 : 5 or 1 : 25. The results (Table 5) demonstrated the satisfactory immunogenic activity of the vaccine.

Summary

1. Sufficiently immunogenic killled vaccines against tick-borne encephalitis can be prepared from virus grown in monolayer cultures of chick embryo cells.

2. Virus can be inactivated with either formalin in a final concentration of 1 : 2,000 or betapropiolactone diluted 1 : 5,000—1 : 10,000.

3. Virus inactivation with formalin has to proceed at 4° C. Inactivation at 37° C fully destroys the immunogenic activity of virus.

4. Tissue culture vaccines protect mice against $10^{4.0}$—$10^{5.5}$ LD_{50} of virus and preserve their activity for 6 months.

5. About 66% of persons having no antibodies before vaccination developed specific antibodies within 4 weeks after vaccination.

References

Daneš, L., Benda, R. (1960*a*): Study of the possibility of preparing a vaccine against tick-borne encephalitis, using tissue culture methods. I. Propagation of tick-borne encephalitis virus in tissue cultures for vaccine preparation. *Acta virol.* **4**, 25.

Daneš, L., Benda, R. (1960*b*): Study of the possibility of preparing a vaccine against tick-borne encephalitis, using tissue culture methods. II. The inactivation by formaldehyde of the tick-borne encephalitis virus in liquids prepared from tissue cultures. Immunogenic properties. *Acta virol.* **4**, 82.

Ilyenko, V. I. (1960): A contribution to the methods of producing a tissue culture formolized vaccine against tick-borne encephalitis. *Acta virol.* **4**, 37.

Levkovich, E. N., and Zasukhina, G. D. (1960*a*): Tissue culture vaccines against tick-borne encephalitis. I. Selection of tissue systems and dynamics of virus inactivation by formalin. *Vop. med. Virusol.* **6**, 33 (in Russian).

Levkovich, E. N., and Zasukhina, G. D. (1960*b*): Tissue culture vaccines against tick-borne encephalitis. II. Examination of immunogenic and antigenic properties. *Vop. med. Virusol.* **6**, 36 (in Russian).

Piskareva, N. A., Pisareva, N. A., and Ivanov, N. P. (1959): The development of the method for the inactivation of antirabic vaccine with ultraviolet rays. I. The influence of different conditions of ultraviolet irradiation on infective and immunogenic properties of fixed rabies virus. *Vop. Virusol.* **4**, 420 (in Russian).

Semenov, B. F., Karaseva, P. S., Rezepova, A. I., and Stepanov, G. M. (1961): Experimental studies of tissue culture vaccine against tick-borne encephalitis. *Vop. Virusol.* **6**, 716. (in Russian).

Smorodintsev, A. A., Kagan, N. V., and Levkovich, E. N. (1941): Experimental data on active immunization against tick-borne encephalitis. *Ž. Mikrobiol. (Mosk.)* **1941** (4), 3 (in Russian). orodintsev, A. A., Levkovich, E. N., and Dankovsky, N. L. (1940): Experiences witth the prevention of spring-summer encephalitis in an epidemic focus by the vaccination of inhabitants with killed virus. *Arch. biol. Nauk* **59** (1—2), 92 (in Russian).

Spitsina, L. N. (1961:: Studies on immunological activity of killed tissue culture vaccine against tick-borne encephalitis. I. Immunological shifts in the vaccinated after the first and second inoculations. *Vop. Virusol.* **6**, 552.

Acta virol. 7 : 61—66, 1963

Pathogenesis of Experimental Coxsackie Virus Infection

Distribution of Coxsackie Virus in Mice after Air-borne Infection*

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Received July 21, 1962

The portal of entry of a virus plays an important role in the study of the pathogenesis of viral diseases. The possibility of aerosol transmission of Coxsackie or ECHO viruses has been reported (Ketiladze and Dreizin, 1960; Tsuker and Leshchinskaya, 1959, and others). We have not found in the accessible literature any experimental studies on the possibility of producing Coxsackie virus infections by aerosol inhalation.

The present paper reports investigations on the course of air-borne Coxsackie virus infection in suckling mice and on the distribution of virus in the organs from infected animals.

Materials and Methods

Coxsackie virus types 4 and 1 of group B, and type 7 of group A were used. The viruses reached titres of $10^{6.3}$—$10^{7.5}$ LD_{50}/0.03 ml. The strains were passaged and titrated in intraperitoneally inoculated newborn mice.

Air-borne infection was performed in a hermetically closed plexiglass chamber constructed according to Prof. S. S. Rechmensky of a 40 litres working capacity under constant conditions: 20—22° C air temperature and 68—73 % relative humidity. Newborn mice were exposed to aerosol in perforated plexiglass cages hanged 10—12 cm. below the top of the chamber.

One ml. portions of the virus suspensions diluted 10 or 20 times were nebulized by a "Chicago" type dispergator. The animals were kept in the chamber for 60 minutes. The dose of virus inhaled by the mice during exposure in the chamber was calculated according to Loosli *et al.* (1943) and was approximately 13,000 intraperitoneal LD_{50}/0.03 ml.

To estimate the minimal dose of Coxsackie virus capable of inducing infection in newborn mice, the concentration of the dispersed suspension was reduced.

The clinical picture of the disease following aerosol infection, the lethality and the presence of virus in tissues from dead animals were studied in the first series of experiments performed in 124 newborn mice. The virus was titrated in the following way: 10% suspensions from carcasses without skin and intestines were diluted with saline in tenfold steps and each dilution was inoculated intraperitoneally into 4—6 one-day-old mice.

In the second series of experiments (118 mice) the animals were killed 20 minutes and 6—10, 24, 48, 72 and 96 hours after aerosol infection. The virus titres were estimated in the lungs, intestines, liver, muscles, brain and

* Read at the Conference on Poliomyelitis, Kiev, 1962.

blood from killed and succumbed newborn mice (organ pools from 4—6 animals). Because circulation of virus could have occurred in the blood from infected animals, blood was removed from the organs of the killed mice by thorough washing with saline by the method of Hirst (1943). A total of 292 mice, old 1—3 days and weighing 1.4—1.8 g. was inoculated.

The *titres of virus* were estimated in newborn mice; in addition to these, monkey kidney cell cultures were used in titrating group B Coxsackie viruses. The highest dilutions causing death of 50% of the animals inoculated, or cytopathic effect in 50% of the monkey kidney cell cultures were considered as titres of virus. Neutralisation tests with type-specific sera were used for the identification of the strains recovered.

Fluorescence microscopy, using acridin orange staining (Maximovich, 1959), was employed for the histochemical assay of nucleic acids in cells infected with Coxsackie virus.

Results and Discussion

Air-borne infection of newborn mice with Coxsackie viruses

Aerosol infection of newborn mice with group B, types 1 or 4, or group A, type 7 Coxsackie viruses resulted in an illness with typical signs of Coxsackie virus infection. The clinical picture of the illness was the same with all the virus strains used, only the incubation period varied somewhat (Table 1).

Table 1. Incubation period of air-borne Coxsackie virus infection

Strain of virus	A-7	B-1	B-4
Survival time of mice (days after inoculation)	2.5—3.5	5—5.6	3—4
Lethality per cents	100	100	100

Disease symptoms appeared the soonest in mice inoculated with Coxsackie A 7 virus. Paralyses of distal extremities occurred within 2.5—3.5 days after inoculation and the animals died 2—20 hours later. Therefore, the incubation period of the illness was practically the same as the survival time of the animals.

Mice inoculated with B 4 and B 1 viruses showed first signs of illness 3—4 and 5—5.6 days after inoculation, respectively. The titres of virus in the carcasses of animals which died or were killed in agony were $10^{6.0}$—$10^{7.3}$ LD_{50}/0.03 ml. Histological examination of the organs from these mice (Brodskaya and Petrovskaya, 1961) revealed changes characteristic of Coxsackie virus infections.

Estimation of the lethal inhalation dose

The dose of virus inhaled was changed by varying the concentration of virus in the dispersed suspension. The lethality and incubation period of the

illness in newborn mice inoculated with various doses of Coxsackie B 4 virus are shown in Table 2. The minimal inhalation dose of virus, causing infection in newborn mice was lower than 1.3—13 intraperitoneal LD_{50}.

Table 2. Inhalation infection of mice with different doses of Coxsackie B-4 virus

Dose of virus inhaled (intraperitoneal LD_{50}/0.03 ml.)	Average incubation period (days)	No. of mice dead out of 10 inoculated
13,000	3.3	10
1,300	3.1	10
130	3.6	5
13	4	3
1.3	4	3

The distribution of Coxsackie virus in organism from icenoculated by inhalation

Our earlier results (Brodskaya and Petrovskaya, 1962) obtained in 3—4 weeks old mice inoculated with Coxsackie virus intraperitoneally, intravenously or by aerosol inhalation showed that the highest lethality occurred with the latter method of inoculation. Signs of disease, however, appeared later in animals inoculated by inhalation than in those inoculated intravenously or intraperitoneally. These findings led us to the assumption that not only adsorption but also multiplication of virus takes place in the lungs. To test this assumption, the following experiments were carried out.

As seen from Table 3, B 1 Coxsackie virus was isolated from the lungs as early as 20 minutes after aerosol inoculation. In the course of the subsequent 1—4 days the titre of virus gradually increased, reaching a maximum ($10^{7.3}$ LD_{50}/0.03 ml.) at the end of the illness. During the first 1—2 days after inoculation no virus could be isolated from any other organ except the lungs. Only 2—3 days after inoculation when the titre of virus in the lungs reached approximately $10^{4.6}$ LD_{50}/0.03 ml., was the virus found in the blood in a titre

Table 3. Distribution of B-1 Coxsackie virus in the organs of 2—3 days old mice inoculated by inhalation of aerosol

Organ	log LD_{50}/0.03 ml. of virus at intervals after inoculation						
	20 min.	9 hrs	1 day	2 days	3 days	4 days	after death
Lung	1.0	1.0	3.7	4.6	4.67	6.0	7.33
Intestines	—	—	—	1.0	3.5	6 37	6.55
Liver	—	—	—	4.0	4.0	7.83	7.5
Brain	—	—	—	—	—	1.0	4.67
Blood	—	—	—	—	3.5	7.33	NT
Muscle	—	—	—	—	2	6.33	5.83

— = no virus detected NT = not tested

of $10^{3.5}$ LD_{50}/0.03 ml. These data indicate that Coxsackie virus, after having sufficiently accumulated in the lungs, enters into the blood stream and is carried to different parts of the host organism.

Viraemia was observed by Melnick (1950), Ho Nan-Chan (1959), Wenner *et al.* (1961) and others after intramuscular, peroral or intraperitoneal inoculation of experimental animals with Coxsackie virus, and by Shelokov and Habel (1957) in a child 5 days before development of clinical signs of illness. The fact that Coxsackie virus was found in the liver somewhat earlier (after 2 days) than in the blood (after 3 days) indicates that small quantities of virus were penetrating from the primary port of entry, i.e. the lungs, into the blood during the whole period following inoculation, but these quantities of virus were not detected by the methods used. The virus gradually accumulates in the liver, as demonstrated by virus titration.

On the 3rd day after inoculation, virus was found in all organs examined, except in the brain; on the 4th day the titres of virus increased by 1—2 log units and it appeared in the brain.

Table 4. Distribution of A-7 Coxsackie virus in the organs of 2—3 days old mice inoculated by inhalation of aerosol

Organ	log LD_{50}/0.03 ml. of virus at intervals after inoculation					
	1 hr.	6 hrs	1 day	2 days	3 days	after death
Lung	—	1.0	4.33	5.5	6.67	7.3
Intestines	—	—	1.33	6.33	7.33	7.33
Liver	—	—	3.5	5.33	7.33	7.5
Brain	—	—	3.0	4.0	5.4	5.5
Muscle	—	—	1.33	6.67	7.5	6.5
Blood	—	—	2.5	6.33	7.5	

— = no virus detected

The gradual increase of the virus titre in the tissues examined, reaching its maximum in animals which died or were sacrificed in agony, indicates that the virus is multiplying in the organs examined. In the intestines the virus appeared only 2 days after inoculation, reaching maximal titres after 4 days. It is to be noted that equally high titres were found on the 4th day in all the other organs examined (with the exception of the brain). The animals died under typical symptoms of Coxsackie virus infection on the 5th day after inoculation. High titres of virus were found in the tissues of animals examined *post mortem*.

The distribution of B 4 Coxsackie virus followed the same pattern. Repeated experiments yielded similar results.

The picture found with group B Coxsackie viruses resembled, in general, that found with group A viruses.

As shown in Table 4, A 7 Coxsackie virus was found in the mouse lungs 6 hours after aerosol inoculation. The titre of virus increased rapidly during the subsequent 3 days, reaching its maximum at death. It is to be mentioned

that following inoculation by aerosol with Coxsackie A 7 virus the infectious process developed very rapidly, the survival time being only 3 days. Therefore it is not surprising that virus could be isolated from the brain, liver, muscles, intestines and blood as early as one day after inoculation, whereas upon inoculation with group B Coxsackie viruses, the latter were not found in these organs earlier than 2—4 days after inoculation.

Two-three days after inoculation of mice with A 7 Coxsackie virus the titre of virus gradually increased, the maximal level being found in the blood ($10^{6.3}$—$10^{7.5}$ LD_{50}/0.03 ml.). The highest amounts of virus were found in the organs of animals which died.

Coxsackie virus isolations from the heart, brain, liver, intestines and lungs from children who succumbed during epidemics of serous meningitis have been reported (Kibrick and Benirschke, 1958; Bozsik, 1959; Dömök *et al.*, 1960).

Examinations of organs from infected mice by fluorescence microscopy revealed that simultaneously with the appearance of virus in tissues, a marked increase in the concentration of ribonucleic acid (RNA) occurred in the cytoplasm of alveolar cells of the lung and of liver and brain cells. Ultraviolet irradiation lasting for 3—5 minutes, sufficient for the polymerisation of the cellular RNA, did not diminish, but, on the contrary, increased the intensity of the fluorescence. This indicates that not only cellular, but also viral RNA s present in the cytoplasm.

Summary

The possibility to cause Coxsackie virus aerosol infection in mice was investigated. The inhalation infection of 2—3 days old mice with B 1, B 4, and A 7 Coxsackie viruses resulted in typical clinical signs and 100% lethality of the inoculated animals.

The study of the distribution of Coxsackie virus in the organs from aerosol-infected mice revealed that the virus is absorbed by lung tissue in which it multiplies; after having reached a high level in the lungs, the virus appears also in the blood, liver, intestines, muscles and brain.

The examination of impression smears of organs from animals showed that together with the appearance of virus in the tissues a marked increase in the concentration of both cellular and viral ribonucleic acid occurs in the cytoplasm of alveolar cells of the lung and of liver and brain cells.

References

Bozsik, G. (1959): Über die histologischen Veränderungen im Nervensystem nach Infektion mit Coxsackie B-3 im Säuglingsalter. *Dtsch. Z. Nervenheilk.* **179**, 564—574.

Brodskaya, E. A., and Petrovskaya, O. G. (1961): Peculiarities of the course of infection with Coxsackie virus in mice inoculated by aerosol. Proc. 6th Sci. Conf. Institute of Poliomyelitis, 179—181 (in Russian)

Brodskaya, E. A., and Petrovskaya, O. G. (1962): The effect of irradiation on the course of infection caused by Coxsackie virus inoculated by different routes. Proc. Conf. "Problems of Radiation Immunology and Microbiology", Moscow, 50—51 (in Russian).

Dömök, I., Molnár, E., and Farkas, E. (1960): Virological investigations into the Bornholm

disease epidemic in Hungary in 1958 and an outbreak of neonatal myocarditis. *Vop. Virusol.* **5,** 321—327 (in Russian).

Hirst, G. K. (1943): Adsorption of influenza virus on cells of the respiratory tract. *J. exp. Med.* **78,** 99.

Ho Nan-chan (1959): Experimental study of B-5 Coxsackie virus in mice and monkey kidney cell cultures. Scientific Bases of the Production of Poliomyelitis Vaccine 1, 302—314, Moscow (in Russian).

Ketiladze, E. S., and Dreizin, R. S. (1960): On some clinical manifestations of illnesses caused by Coxsackie viruses. *Sovetsk. Med.* **2,** 79—82 (in Russian).

Kibrick, S., and Benirschke, K. (1958): Severe generalized disease (encephalomyocarditis) occurring in the newborn period and due to infection with Coxsackie virus group B. Evidence of intrauterine infection with this agent. *Pediatrics* **22,** 857—875.

Loosli, C., Robertson, O., and Puck, T. (1943): The production of experimental influenza in mice by inhalation of atmospheres containing influenza virus dispersed as fine droplets. *J. infect. Dis.* **72,** 142—153.

Maximovich, N. A. (1950): Investigations on intranuclear inclusions in viral influenza by fluorescence microscopy method. *Doklady Akad. Nauk SSSR* **128,** 1286—1289 (in Russian).

Melnick, J. L. (1950): Studies on the Coxsackie viruses. Properties, immunological aspects and distribution in nature. *Bull. N.Y. Acad. Med.* **26,** 343—356.

Shelokov, A., and Habel, K. (1957): Viremia in Coxsackie B meningitis. *Proc. Soc. exp. Biol. (N.Y.)* **94,** 782—784.

Tsuker, M. G., and Leshchinskaya, E. V. (1959): Clinical and epidemiological peculiarities of epidemics of serous meningitis. Proc. 2nd Sci. Session of the Institute of Infectious Diseases, 66—68 (in Russian).

Wenner, H. A., Te Jong, L., and Kamitsuka, P. (1961): Experimental infections with Coxsackie viruses. *Arch. ges. Virusforsch.* **10,** 426—450.

Acta virol. **7 : 67—75, 1963**

Evaluation of the Efficiency of Tobacco Mosaic Virus Purification Procedures by the Polarographic Method

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Received June 27, 1962

Electrophoretic homogeneity has been considered a standard criterion of the absence of non-virus proteins from a purified virus preparation. Preliminary experiments showed that tobacco masaic virus (TMV) preparations homogeneous upon electrophoresis still contain non-virus proteins exhibiting a strong polarographic activity. This result could be expected as even in the most ideal case the presence of 1 per cent of contaminating proteins can be detected by the electrophoretic method only with difficulties, whereas polarographically 0.2% of non-virus protein in a TMV preparation can be detected rather easily (Ruttkay-Nedecký, 1957*a*, 1963). After the non-virus protein content of viral preparations had reached a concentration lower than several per cents it is impossible to follow the effects of further purification procedures electrophoretically.

In the present work the polarographic method was used for comparing the effectiveness of two purification methods: the chemical (Bawden, 1950) and differential high-speed centrifugation methods.

Materials and Methods

Purification of TMV. Tobacco leaves infected with a common strain of TMV were used as starting material. A K_2HPO_4 solution was added to the expressed juice to reach a final concentration of 0.01 M. Unsoluble material and the precipitate formed were separated by centrifugation at 3000 × g for 30 minutes. The supernatant fluid thus obtained designated IJ, represented the starting infective juice, one part of the juice destined for polarographic analysis and for protein nitrogen determinations was dialyzed for 2 days in motion against 0.1 M phosphate buffer, pH 7. The major part of the juice was divided into two portions, A and B. Portion A was subjected to chemical purification and portion B only to differential centrifugation. The course of the two purification procedures used (A and B) and the designation of the individual steps are schematically illustrated in Table 1. Procédure A was similar to that suggested by Bawden and Pirie (see Bawden, 1950), except that isoelectric precipitation was followed by dialysis against buffer solution instead of dialysis against tap and distilled water as proposed by the authors mentioned.

To establish whether particles sedimenting under the experimental conditions used were present in uninfective juice, a control purification C (Table 1) was included. Healthy tobacco leaves were subjected to the same procedure as infected leaves and the starting uninfective juice (designated NJ) was subjected to two cycles of centrifugation as in B, the quantitative ratios for resuspending of the sediment having been kept the same. The sample was not treated further, because it neither showed polarographic activity, nor could protein nitrogen be detected in it.

Non-virus proteins of the IJ are represented by sample B-Sc, the supernatant fluid obtained after high speed centrifugation of the IJ. The corresponding control sample N-Sc was obtained in the same manner by high speed centrifugation of the NJ. Prior to the assays all the samples were either dissolved in or dialyzed against 0.1 M phosphate buffer pH 7.

The starting juice, the partially purified products and the final preparations (see Table 1) were polarographed at various concentrations expressed in mg of protein nitrogen per 100 ml. (mg%N) assayed by the Kjeldahl method.

Polarographic analyses were carried out at 0° C as previously described (Ruttkay-Nedecký, 1963). A supporting electrolyte composed of 0.001 M $Co[NH_3]_6Cl_3$, 0.1 M NH_4Cl and 0.1 M NH_4OH pH 9.5 (measured at 25° C), and designated further on s. el. 9.5, was used. A polarograph LP 55 equippped with a galvanometer of a sensitivity of 2.6×10^{-9} A per mm × m, was employed. The rate of mercury flow was 1.27 mg./sec., and the drop time 4.7 sec. (in 0.1 M KCl, the electrodes short circuited). The curves were registered from —0.8 V with a potential drop of 200 mV/abc. and a sensitivity s = 1/100. The time which elapsed from the moment of contact of the sample with the supporting electrolyte to the beginning of the recording corresponded to about 4 minutes. The heights of the first or second part of the protein double wave were measured from the cobalt reduction wave on. The heights of the waves were recalculated to μA and designated P_1 and P_2 for the first and second wave, respectively. The concentration of proteins c was expressed in mg%N.

Electrophoretic assay. The purification procedures A and B were checked electrophoretically with an Antweiler-type microelectrophoretic apparatus (Boskamp Geräte-Bau K. G.) with interferometric evaluation 0.1 M phosphate buffer pH 7 and current intensity of 1.5 mA were used, the time of movement being 15 minutes.

Infectivity control. The final TMV preparations caused necrotic lesions on leaves of *Nicotiana tabacum* L. cultivar Xanthi in a concentration as low as 10^{-10} g./ml.

Results and Discussion

Evaluation of the efficiency of the purification procedure by the electrophoretic method

It was impossible to evaluate the course of purification electrophoretically, because even the concentrated virus preparation A-TMVa_2 obtained after the first step of purification showed but one component on electrophoresis.

The electrophoretic curve of the sample A-TMVa_2 is presented in Fig. 1 (curve 1). As its asymmetry was not reduced after further chemical purification, i.e. by precipitation at the isoelectric point and by dialysis, but as it was abolished after high-speed centrifugation, it appears that this asymmetry was caused by the presence of fragments and aggregates of virus particles rather than by accompanying proteins. The sample A-TMVg_2 yielded an electrophoretic curve showing neither traces of an unhomogeneity nor the presence of another component (Fig. 1, curve 2). The samples B-TMVg_2, B-TMVg_4 and B-TMVg_6 from the purification procedure B also were electrophoretically homogeneous.

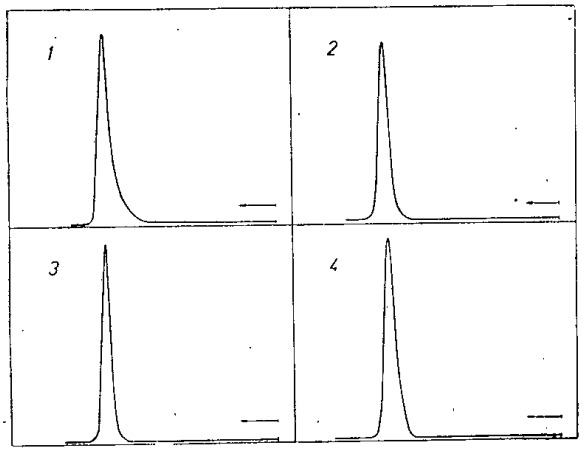

Fig. 1.
Electrophoretic curves (interferometric records) of tobacco mosaic virus preparation (see Table 1)
Current intensity 1.5 mA, time of movement 15 minutes, 0.1 M phosphate buffer of pH7.
1 — A-TMVa_2; 2 — A-TMVg_2; 3 — A-TMVg_4; 4 — B-TMVg_6.

The electrophoretic curves of both final purified samples A-TMVg_4 and B-TMVg_6 are presented in Fig. 1 (curves 3 and 4). IJ could not be assayed electrophoretically because of its low protein content and dark colour.

Evaluation of the efficiency of the purification procedure by the polarographic method

Polarographic records of the purification procedures A and B are presented in Figs 2 and 3 (see plates).

The infective juice (sample IJ, Fig. 2) gave polarographic curves typical

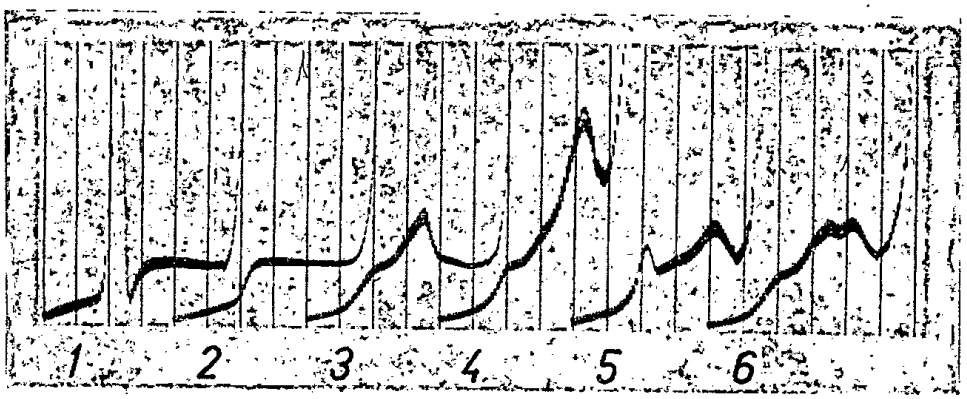

Fig. 4.

Comparison of the polarographic effects of TMV, non-virus protein and a mixture of the two at 0° C in s.e. 9.5

1 — standard electrolyte pH 9.5; 2 — standard electrolyte pH 9.5 plus gelatine; 3 — TMV (52.1 mg%N); 4 — non-virus protein (1.5 mg%N); 5 — non-virus protein (0.3 mg%N); 6 — TMV (52.1 mg%N) + non-virus protein (0.3 mg%N). The curves were registered at 0° C from —0.8 V with a potential of 0.2 V/abc.

of non-virus proteins. Similar curves were obtained on polarography of the supernatant fluid obtained after high-speed centrifugation of infective juice (sample B-Sc, Fig. 3). The final preparations A-TMVg_4 and B-TMVg_6 gave polarographic curves typical of TMV and the sample A-TMVi (Fig. 2) yielded double wave curves, the first wave belonging to the virus and the second to non-virus proteins.

Polarographic curves of the final preparations of TMV and of non-virus protein are compared in Fig. 4. The first curve belongs to the supporting electrolyte, the 2nd to the same electrolyte but with gelatine added to suppress the cobalt maximum, the 3rd to TMV, the 4th and 5th curves to non-virus protein polarographed at two different concentrations (sample NJ), and the last curve to a mixture of TMV with non-virus protein in a w/w ratio 170 : 1. As can be seen from Fig. 4, the peak of the virus curve was at a potential of —1.5.V, the peak of the non-virus protein curve being —1.65.V. The potentials of —1.5.V and —1.65 V are those of the peaks of the first and second part of the

protein double wave if carrying out polarography in s. el. 9.5 at 0° C, i.e. under the conditions used in the present work. The agreement of potentials under identical conditions is a sufficient reason for identifying the wave of the virus with the first part of the usual protein double wave (Brdička, 1933) and the wave of the non-virus proteins with the second part of this double wave. The second part of the double wave is practically absent from polarographic records of thoroughly purified samples of TMV. But polarographic curves of the non-virus protein practically show only the second part of the double wave, because, as compared with the second peak, the first part of the wave is so low that it is covered by the rising branch of the second wave. This characterization of the virus and non-virus protein waves is valid only at 0° C.

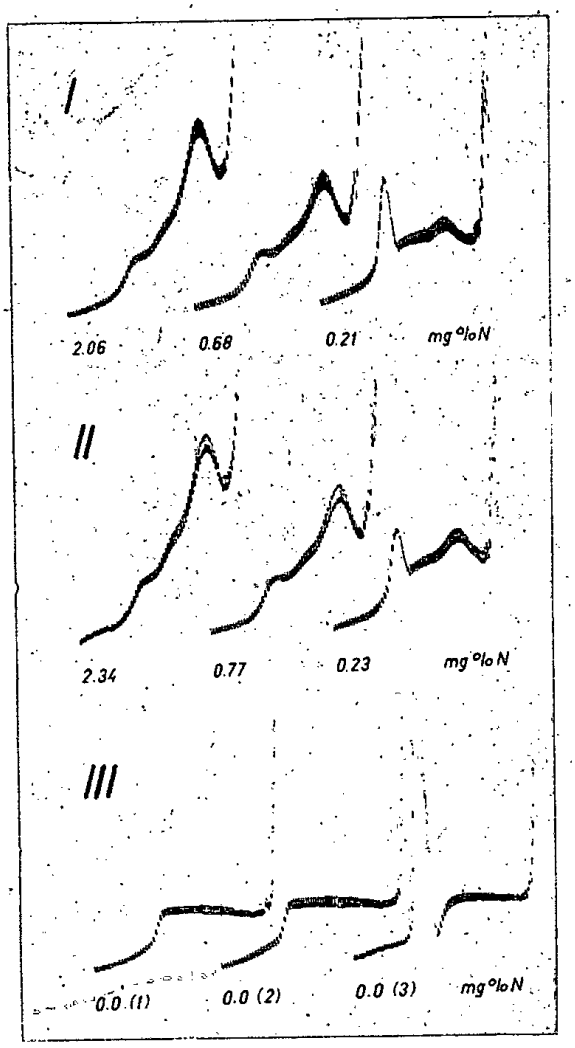

Fig. 5.

Polarographic curves of control samples polarographed at 0° C in s.e. 9.5 at varying concentrations (indicated below the curves)
I — NJ; II — N-Sc; III — Ng_2; 1 — twofold, 2 — fourfold dilution, 3 — supporting electrolyte

Non-infected material (control)

Polarographic curves of the non-infected juice (NJ), of the supernatant fluid after high-speed centrifugation of the NJ (N-Sc), and of the sample Ng_2 are presented in Fig. 5. The samples NJ and N-Sc gave the same curves as the samples IJ and B-Sc, i.e. curves typical of non-virus proteins. The sample Ng_2 did not yield a polarographic protein wave even after fourfold (curve 2) or twofold (curve 1) dilution. The sample B-TMVg_2 (corresponding to sample Ng_2) concentrated in the same ratio with respect to the starting juice as Ng_2, contained TMV in a concentration of 248 mg%N and was polarographically active even after a hundredfold dilution. No protein nitrogen could be demonstrated by the semimicro-Kjeldahl method in sample Ng_2, as this contained less than 0.1 mg%N. The control purification showed that under the conditions used practically no sediment is obtained from healthy tobacco juice.

Comparison of the efficiency of two purification procedures

The maximal purity of a virus preparation can be characterized by the absence of the second part of the polarographic protein double wave. In purified preparations containing nonvirus proteins the height of the second part of the double wave reflects the extent of contamination with non-virus proteins (Ruttkay-Nedecký, 1957*a*, 1963). It is therefore advantageous to compare the purification efficiency on the basis of the depression of the second wave. This

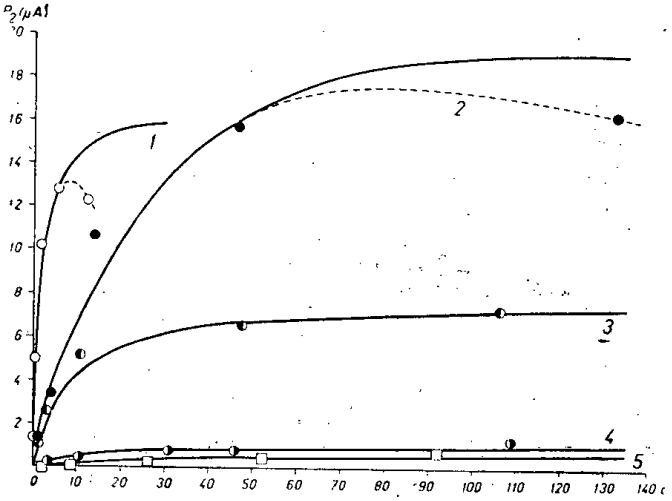

Fig. 6.

Relationship between the height of the second wave (P_2) of the protein double wave and the concentration (c) in the course of purification procedure A

1 — IJ; 2 — A-TMVa_2; 3 — A-TMVi; 4 — A-TMVg_2; 5 — A-TMVg_4

The full lines represent the relations (1) with the coefficients a and b chosen to suit the experimentally established values. The deviation from the approximated relation (1) is marked by a dashed line.

depression can be seen more clearly from the graphical presentation in Figs 6 and 7 than from Figs. 2 and 3. The graphs on Figs 6 and 7 show the relationship between the height of the second part of the protein double wave and the concentration of the polarographed samples, as obtained in the course of the purification procedures A and B.

The relationship between the height of the second wave and the concentration (Figs 6 and 7) is approximated by the relation (1):

$$P_2 = \frac{c}{a \times c + b} \qquad (1)$$

where P_2 is the height of the second part of the polarographic double wave in μA, c the concentration in mg%N and a and b are empirical coefficients

(Ruttkay-Nedecký, 1960). The full lines on the graphs are graphical presentations of relation (1) with the coefficients a and b chosen to suit the experimentally established values. The approximation was calculated up to the concentration belonging to the maximal height of the second wave. At concentrations higher than this the height of the wave decreases. The deviation from the approximated relation is symbolized by a dashed line.

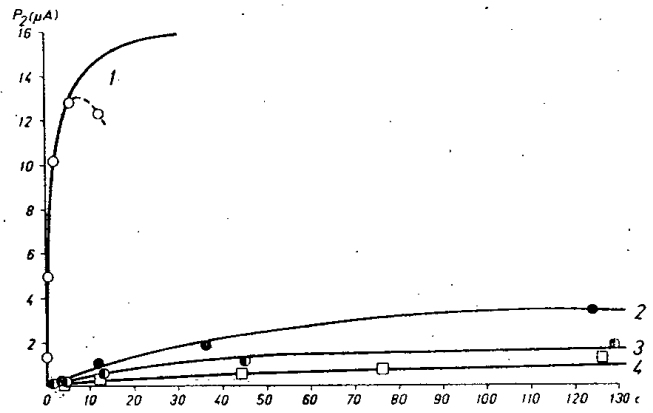

Fig. 7.

Relationship between the height of the second wave (P_2) of the protein double wave and the concentration (c) in the course of purification procedure B

1 — IJ; 2 — B-TMVg_2; 3 — B-TMVg_4; 4 — B-TMVg_6
For other explanations see Fig. 6.

It is evident from Figs 6 and 7, that, as compared with the whole chemical part of purification procedure A, two cycles of high-speed centrifugation of the infective juice in procedure B caused an uncomparably more pronounced flattening of the dependence of the height of the second wave on concentration, i.e. a better purification of the virus resulted. Six cycles of high-speed centrifugation in procedure B were equally efficient as the combination of chemical purification and four cycles of diferential centrifugation. Both purification procedures A and B yielded virus preparations (A-TMVg_4 and B-TMVg_6) which did not differ in their polarographic properties. This is evident from Fig. 8 presenting the relationship between the height of the first and second waves and the concentration of the final preparations A-TMVg_4 and B-TMVg_6. Using for both samples the points, established experimentally it is possible to draw a single curve. Similarly to other figures, the full lines in Fig. 8 represent the graphically expressed relation (1) with coefficients a and b chosen to suit the experimentally established values. The deviation from the approximated curve is presented as a dashed line. For comparison also the dependence of the height of the second wave on the concentration of non-virus proteins is presented (curve III). The experimentally established values were obtained by examining samples NJ and N-Sc obtained from non-infected juice. The steep dependence found with non-virus proteins is in sharp contrast

with the very flat dependence of TMV (curve II). On the polarographic curves of the final preparations (A-TMVg_4 and B-TMVg_6) a scarcely noticeable second wave was observed at very high concentrations (Figs. 2 and 3). The height of these waves, i.e. the P_2 values were 0.8 μA at a concentration of

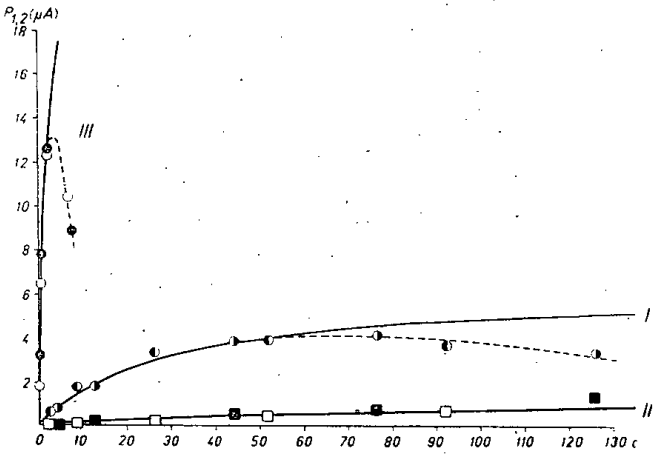

Fig. 8.

The concentration dependence of the height of the first (I) and second (II) wave of the protein double wave of TMV and of the second wave of non-virus proteins (III)
TMV: samples A-TMVg_4 (◐, □) and B-TMVg_6 (◑, ■).
Non-virus proteins: samples NJ (○) and N-Sc (●).
For other explanations see Fig. 6.

Table 1. Schematic representation of the procedures used for the purification of TMV (A and B) and for the treatment of uninfective juice (C)

A

Infective juice (IJ)

↓

Two cycles of precipitation with 250 g./l. ammonium sulphate and dissolution in distilled water

(A-TMVa_2)

↓

Precipitation of virus at pH 3.4, dissolution in 0.1 M phosphate buffer pH 7 and dialysis for 2 days at 4° C against the same buffer. Unsoluble material removed by low-speed centrifugation

(A-TMVi)

↓

Two cycles of low (10,000 rev/min. for 30 mins) and high (25,000 rev/min., about 50,000 × g, for 2 hrs) speed centrifugation. The pelleted virus dissolved in 0.1 M phosphate buffer pH 7

(A-TMVg_2)

↓

Another two cycles of low and high speed centrifugation under the same conditions

(A-TMVg_4)

B

Infective juice (IJ)

↓

Two cycles of low (10 000 rev/min. for 30 mins) and high (25,000 rev/min., about 50 000 × g, for 2 hrs) speed centrifugation. The pelleted virus dissolved in 0.1 M phosphate buffer pH 7

(B-TMVg_2)

↓

Two further cycles of low and high speed centrifugation

(B-TMVg_4)

↓

Another two cycles of low and high speed centrigugation

(B-TMVg_6)

C

Healthy (normal) juice (NJ)

↓

Two cycles of low and high speed centrifugation as in B

(Ng_2)

92 mg%N and 1.3 μA at a concentration of 126 mg%N for A-TMVg_4 and B-TMVg_6, respectively. The reciprocal value of the coefficient b from equation (1), designated rb_2 for the second wave (Ruttkay-Nedecký 1957*b*, 1960) equals the limit of the ratio P_2/c for c approaching zero. At low concentrations of the non-virus protein, the value of rb_2 therefore expresses the specific polarographic activity of this protein in μA/mg%N. For the non-virus proteins NJ and N-Sc the rb_2 value experimentally established was 11.1 μA/mg%N. Using this value for recalculation of the P_2 value for the amount of non-virus proteins in samples A-TMVg_4 and B-TMVg_6 we find that the content of non-virus protein impurities in either preparation did not exceed 0.1%.

After reaching a certain concentration, a decrease of the wave is observed upon further increase of the concentration of TMV. With TMV this phenomenon can be observed on the first wave only, whereas with other proteins both waves are decreased. This phenomenon was discussed in another paper (Ruttkay-Nedecký, 1960). On polarographic curves of purified virus preparations the presence of the virus causes also a decrease or disappearance of the cobalt maximum.

Although in the samples of the first two steps of purification procedure A, i.e. in the concentrated virus preparations A-TMVa_2 and A-TMVi (Fig. 2) no non-virus proteins could be detected electrophoretically; the polarographic curves of these samples still exhibited a considerable polarographic activity belonging to non-virus proteins. In this case the advantages of polarography over the standardly used electrophoretic method were clearly shown.

Summary

Purified tobacco mosaic virus (TMV) preparations, in which not more than 0.1% of non-virus proteins (calculated with respect to the virus) could be detected by the polarographic method were obtained by repeated differential centrifugation of infective tobacco juice without the use of chemical purification. Two cycles of differential centrifugation of the infected juice remove more non-virus proteins than a combination of two cycles of precipitation with ammonium sulphate and precipitation at the isoelectric point followed by dialysis against 0.1 M phosphate buffer. A virus purified by this chemical procedure and subsequently by four cycles of differential centrifugation showed the same properties as virus purified by six cycles of differential centrifugation of the same starting material. The polarographic method proved to be much more sensitive than the electrophoretic method for comparing purification procedures and for the assay of non-virus proteins in purified TMV preparations.

Acknowledgement. Thanks are due to Mrs. K. Chmulíková and Miss M. Hronská for technical assistance.

References

Bawden, F. C. (1950): *Plant viruses and virus diseases.* 3rd ed. Chronica Botanica Co., Waltham, Mass., U.S.A., p. 172.

Brdička, R. (1933): A new test for proteins in the presence of cobalt salts in ammoniacal solutions of ammonium chloride. *Collection Czechoslov. Chem. Commun.* **5**, 112—128.

Ruttkay-Nedecký, G. (1957*a*): A polarographic method for detection of protein impurities in preparations of tobacco mosaic virus. *Biochim. biophys. Acta* **26**, 455—456.

Ruttkay-Nedecký, G. (1957b): Polarographic activity of tobacco mosaic virus protein and the use of the polarographic method for the indentification of protein impurities in purified virus preparations. *Acta virol.* **1**, 21—29.

Ruttkay-Nedecký, G. (1960): Charakteristische polarographische Aktivität des Tabakmosaikvirus. I. Unterschied im polarographischen Effekt des Virus und der Nicht-Virus-eiweissstoffe. *Collection Czechoslov. Chem. Commun.* **25**, 3363—3379.

Ruttkay-Nedecký, G. (1963): Charakteritische polarographische Aktivität des Tabakmosaikvirus. III. *Collection Czechoslov. Chem. Commun.*, **28**, 585—594.

Acta virol. 7 : 76—81, 1963

Virological and Serological Investigations of Sporadic Cases of Serous Meningitis

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Received July 4, 1962

During the last years there have been many reports about the role of enteroviruses in the etiology of serous aseptic meningitis. At least 28 different serotypes of enteroviruses have been related with this disease. The majority of investigations dealt with epidemic outbreaks. Of especial interest, however, is the elucidation of the etiology of sporadic cases of serous meningitis. Such studies have been carried out by Habel *et al.* (1957), Davis and Melnick (1958) and Godtfredsen (1959). These authors based their diagnosis of enterovirus infection either on virus isolation or on the virus isolation supported by an antibody increase against the serotypes isolated. Yampolskaya and Zalmanzon (1961) examined serologically a limited number of patients using a broad scale of various enteroviruses.

The present paper deals with the role of enteroviruses and of mumps virus in sporadic cases of serous meningitis which have occurred in Leningrad from 1958—1961. The years 1958 and 1959 differed distinctly from 1960—1961 in the incidence of poliomyelitis. As a result of the mass vaccination against poliomyelitis with Sabin's live vaccine of all inhabitants of Leningrad aged up to 20 years cases of poliomyelitis practically disappeared, which influenced markedly the etiological pattern of serous meningitides.

Results are also reported of extensive serological examinations with enteroviruses employed in cases of serous meningitis in which virus isolation was unsuccesful.

Materials and Methods

Virus isolation was attempted from 57 samples of spinal fluid taken during the first week of disease and from 167 stool samples taken from 79 patients at various time intervals after the onset of serous meningitis.

Monolayer cultures of human embryo fibroblast and stable human amnion cells were used. Ten per cent stool suspensions were subjected to two cycles of freezing and thawing and centrifuged at 1000 and 5000 rev/min. Tube cultures were inoculated with 0.5—1.0 ml. of the examined material, stored previously at —10° C. The medium from the primary inoculated cultures was harvested on the 7th—8th and 12th—14th day of incubation at 35° C and inoculated into fresh cell cultures.

Spinal fluids and stools negative in cell cultures were tested in newborn mice old 24 or, exceptionally, 48 hours. Each animal was inoculated both intracerebrally and intraperitoneally with 0.2 and 0.4 ml. of the materials, respectively. Two blind passages were made 7—8 days after primary inoculation, using 10% carcass suspensions.

The viruses isolated were titrated and identified in monolayer cell cultures. Cytopathic agents were identified according to the neutralization of the cytopathic effect with antisera against types 1, 2 and 3 of poliovirus, types Bl-5 and A9 of Coxsackie virus and types 1—14, 17—20 and 25—27 of ECHO virus. The sera were obtained from the Moscow Institute of Viral Preparations.

Serological examinations involved 71 paired sera (sera from 8 patients with positive virus isolation were nor examined). The first sample was taken in the acute phase of disease and the

second 2—3 weeks later during convalescence. Both serum samples were heated at the same time at +56° C for 1 hour. All the sera were examined for their antibody contents against types 1, 2 and 3 of poliovirus. The rise of antibodies against the strain isolated was determined.

Where virus isolation and antibody increase to polioviruses were negative, additional serological examinations were done. First a haemagglutination-inhibition (HI) test with mumps virus was made to exclude this virus as a possible etiological agent. In negative cases the sera were then assayed in neutralization tests with various enteroviruses, first with ECHO type 4, 6, 9 and Coxsackie A9 viruses, then with the Bl-5 Coxsackie group, and finally with ECHO virus types 1, 2, 3, 5, 7, 11, 12, 14 and 16.

With enteroviruses the neutralizing capacity of the sera was examined against 100 TCD_{50} of virus. Stock strains of enteroviruses were obtained from the Department of Virology of the Institute of Experimental Medicine, U.S.S.R. Academy of Medical Sciences. Tests with polioviruses and Coxsackie B viruses were done in stable human amnion cells, and with ECHO viruses in trypsinized human embryo fibroblast cell cultures. The HI test with mumps virus was performed with an allantoic strain obtained from the Pasteur Institute of Epidemiology and Microbiology, Leningrad. The test was carried out at 20—22° C with 4 haemagglutinating units of virus.

In all serological tests, an at least fourfold increase of the antibody titre was considered significant. If the increase was only fourfold, the test was always repeated.

Results

Seventy-nine persons showing the syndrome of serous meningitis were examined. Their age was as follows:

Age of the patients (years)	0—3	4—7	8—11	12—15	16 and over
Number of patients examined	12	22	25	10	10

The clinical picture showed a uniform course and was characterized by a sudden onset, intensive cephalalgia, vomiting, rise of temperature to 38 to 40° C und meningeal symptoms. The spinal fluid in all cases was pathologically changed. The cell number varied between 75/3 to 4032/3 and the protein concentration was from 0.016—0.66 mg%. In spite of the great variation in the cell numbers in the spinal fluid, an analysis in retrospect of the cell number and protein content made it impossible to refer the illness to a certain etiological group. There were no characteristic changes in the blood.

Virus isolation was positive from 41 persons and all the viruses were isolated

Table 1. Results of virus isolation experiments and of tests for antibody increase against the viruses isolated in 41 patients

Number of patients with	Viruses isolated										
	Polio	Polio and E-25	Coxsackie A 9	Coxsackie B 2	ECHO 4	ECHO 6	ECHO 9	ECHO 20	Adeno	Not identified	Total
Positive virus isolation	7	1	3	1	9	2	11	1	1	5	41
Antibodies in the 2nd serum sample examined	4	1	3	1	7	2	8	1	1	5	33
Antibody increase	2	0	3	1	6	2	7	0	1	3	25

in cell cultures. Materials from 38 patients, negative in cell cultures, were inoculated into newborn mice without causing a disease in them.

From the spinal fluids of 4 persons, type 4 ECHO virus was isolated. From the stools of 40 persons cytopathic agents were obtained (Tab. 1), namely polioviruses from 7, Coxsackie viruses from 3 and ECHO viruses from 23 persons. From one child type 25 ECHO virus was isolated on the 5th day and type 2 poliovirus on the 8th day of illness. In five cases the viruses could not be identified with the sera at our disposal. From the stools of one person an adenovirus was isolated.

An increase of antibodies to the viruses isolated and to polioviruses was found in paired sera from 32 persons. In cases in which viruses other than those of the polio group were isolated, there was no antibody increase against polioviruses.

In the group with positive isolations of Coxsackie and type 4, 6 and 9 ECHO viruses, positive serological results were obtained with 19 out of 21 persons.

Paired sera from a patient from whom type 20 ECHO virus was isolated, showed practically no antibodies against the strain isolated (titre 1 : < 10). In the case of positive isolation of type 25 ECHO virus and type 2 poliovirus, no increase of antibodies against the agents isolated could be demonstrated. The titre against type 25 ECHO virus was 1 : 10 and that against poliovirus 1 : 80 in both sera. This case was referred to the group of poliomyelitis patients.

In three out of 5 persons from whom not identified viruses were isolated, a rise of antibodies against the agents isolated was demonstrated; in one case there was no antibody increase to the strain isolated (titre 1 : < 10); and in the fifth person a twofold decrease of the antibody titre to the strain isolated was found together with an increase of the level of anti-mumps virus haemagglutinins.

From two stools of one child an adenovirus strain was isolated on the 17th and 30th day of illness. During the first two weeks virological examinations were negative in this child. Although the antibody titre on the 7th and 29th days was 1 : 10 and 1 : 80 respectively, the late isolation of adenovirus made doubtful its etiological role in the given illness. Subsequent examinations of blood serum revealed an 8-fold increase of antibodies against Coxsackie B2 virus (1 : 160 and 1 : 1280).

Table 2. Results of serological examinations of patients, from whom either no viruses, or an adenovirus or ECHO 20 virus or not identified agents (against which there was no antibody increase) were isolated

Number of patients								
With antibody increase against viruses							With no antibody increase	Total
Polio	ECHO			Coxsackie		Mumps		
	9	6	5	B 2	B 3			
3	3	1	1	1	1	9	24	43

Table 3. Etiology of serous meningitides occurring in Leningrad from 1958—1961

Year	Polio virus	ECHO				Coxsackie			Not identified viruses	Mumps virus	Etiology obscure	Total of persons examined
		4	5	6	9	A 9	B 2	B 3				
1958	6	—	—	—	—	—	—	—	—	—	1	7
1959	1	1	—	1	2	1	—	—	—	—	4	10
1960	1	3	—	1	8	2	1	—	2	3	9	30
1961	2	5	1	1	4	—	1	1	1	6	10	32
Total	10	9	1	3	14	3	2	1	3	9	24	79

In 38 persons the isolation experiments were negative. Their paired sera were tested serologically as described above. In addition, sera from 5 persons were examined, from whose stools were isolated cytopathic agents, the etiological role of which was doubtful (ECHO 20, adenovirus and unidentified viruses which did not cause an antibody increase).

In 10 cases an increase of antibodies against enteroviruses was present. In 9 persons there was a rise of antibodies against mumps virus; in these cases the illness had the form of an isolated serous meningitis without involvement of the parotis or other glands. In 24 patients the antibody titres in the first and second blood samples did not differ (Tab. 2). It must be mentioned that the clinical picture in this group of persons differed in no way from serous meningitis caused by enteroviruses.

The summarized results of virological and serological investigations on persons suffering from serous meningitis (Tab. 3) show that in 43 out of 79 patients, i.e. in more than a half of the cases, enteroviruses were the causative agents. The incidence of meningitides caused by enteroviruses was the same in all age groups. Serous meningitis caused by mumps virus accounted for 1/9 of the cases. In about 1/3 of the patients the etiology of the illness could not be determined.

Discussion

A comparison of the results of virological (Tab. 1) and serological (Tab. 2) examinations of persons with enteroviral meningitides shows that virus isolation is a more valuable diagnostic method than the demonstration of an antibody increase with negative virological findings. Neutralisation tests with many serotypes of enteroviruses confirmed the etiology only of 1/4 (10 cases) of illnesses shown to be caused by enterovirus infection. But it should be stressed that isolation of a virus from the stools is of diagnostic value only in those cases, in which the etiological role of the agent isolated in the given disease is unequivocal.

On the other hand, when isolating an agent whose bearing on human pathology has not yet been elucidated, the determination of antibodies against the agent isolated is of decisive significance. The same concerns the elucidation of the role of viruses of an unknown group pertinence. We found that when there was no rise of antibodies against the unknown agent isolated further examinations were necessary to elucidate the actual cause of serous meningitis.

Of especial interest was the isolation of an adenovirus accompanied by a simultaneous rise of antibodies to the strain isolated and Coxsackie B2 virus. The isolation of adenoviruses from persons with serous meningitis has been reported (Habel *et al.*, 1957), but their etiological role in this disease remains obscure. Therefore in every case of serous meningitis with positive isolation of an adenovirus the exclusion of any other possible causes is unavoidable.

It is evident from Table 3 that in 1958 (up to the application of live polio vaccine) there occurred mainly serous meningitides caused by poliovirus. In 1960—1961 the etiological pattern of the disease changed and type 4 and 9 ECHO viruses were encountered the most frequently as etiological agents. Other authors also found a prevalence of certain serotypes of enteroviruses in sporadic cases of serous meningitis. Davis and Melnick (1958) found that 62% of the cases were caused by type 1 poliovirus and ECHO 6 virus (in equal proportions). According to Godtfredsen (1959), ECHO 9 virus was the most frequent cause of enterovirus meningitides. It is interesting that types 4, 6 and 9 of ECHO viruses have been reported to cause epidemic outbreaks of serous meningitis (Malherbe and Harwin, 1957; Karson, 1957; Davies *et al.*, 1958; Henessen, 1957; and others). Other types of ECHO viruses have been encountered almost exclusively in sporadic cases of the disease.

Summary

Results of virological and serological investigations on 79 persons suffering from the syndrome of serous meningitis (sporadic cases) are presented. The examinations done in Leningrad from 1958—1961 revealed the considerable importance of enteroviruses in the etiology of the disease. Enteroviral nature of the disease was proved in more than a half of the cases.

In 1958 most of the serous meningitides were caused by polioviruses. In 1960—1961 the majority of infections were caused by type 9 and 4 of ECHO virus. Serological examinations with a broad spectrum of enteroviruses made possible a diagnosis of only 1/4 of those cases, in which no virus was isolated.

Tests for antibodies against mumps virus proved to be very important.

Human embryo fibroblast cells and a stable line of human amnion cells, supplementing each other, proved very suitable for demonstrating the enteroviral etiology of serous meningitides. Virus isolation in suckling mice was unsuccessful.

References

Davies, J. W., McDermott, A., and Severs, D. (1958): Epidemic virus meningitis due to ECHO-9 virus in Newfoundland. *Canad. med. Ass. J.* **79**, 162—167.

Davis, D., and Melnick, J. L. (1958): Poliomyelitis and aseptic meningitis. A two-year field and laboratory study in Connecticut. *J. Lab. clin. Med.* **51**, 97—117.

Godtfredsen, A. (1959): Strains of Coxsackie virus type A-9 and ECHO virus type 9 recovered from cerebrospinal fluids. *Acta path. microbiol. scand.* **46**, 217—222.

Habel, K., Silverberg, E. J., and Shelokov, A. (1957): Isolation of enteric viruses from cases of aseptic meningitis. *Ann. N.Y. Acad. Sci.* **67,** 223—229.

Henessen, W. (1957): Untersuchungen über das Virus der epidemischen meningitis (ECHO-Virus 9). *Z. Hyg. Infekt.-Kr.* **144,** 125—147.

Karson, E. T. (1957): Outbreaks of aseptic meningitis caused by type 6 ECHO virus. Excerpta Medica. Fourth international poliomyelitis conference. Geneva, 1957, 8.

Malherbe, H., and Harwin, R. (1957): An outbreak of aseptic meningitis associated with ECHO-virus type 4. *S. Afr. med. J.* **31,** 1261—1264.

Yampolskaya, E. I., and Zalmanzon, E. S. (1961): To the clinical and virological characteristics of serous meningitis caused by enteric (Coxsackie and ECHO) viruses. *Pediatrija* **1961** (4), 19—26 (in Russian).

Acta virol. 7: 82—83, 1963

Report on the Isolation from *Ixodes persulcatus* Ticks and from Patients in Western Siberia of a Virus Differing from the Agent of Tick-borne Encephalitis

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Received October 9, 1962

In May—July, 1962, a second scientific expedition led by Prof. M. P. Chumakov (chief of the epidemiological section was Dr. G. S. Sarmanova) and organized by the Institute of Poliomyelitis and Viral Encephalitides of the U.S.S.R. Academy of Medical Sciences carried out investigations in natural foci of tick-borne encephalitis in the Kemerovo region. Thirty-seven workers of the above Institute, 11 workers of the Kemerovo Regional Sanitary-Epidemiological Station, and 6 workers of the Institute of Virology of the Czechoslovak Academy of Sciences in Bratislava led by H. Libíková, participated in the expedition.

One of the aims of this joint Soviet—Czechoslovak expedition was the virological investigation of Ixodid ticks, vectors of tick-borne encephalitis, as well as of blood specimens from both patients suspected of being infected with tick-borne encephalitis virus and healthy persons bitten by ticks. This material was assayed in parallel at three laboratories of the expedition by inoculating newborn and adult white mice, chick embryos, and cultures of chick embryo and pig embryo kidney cells. Thanks to the variety of simultaneously employed methods the workers succeeded in isolating not only a number of typical strains of tick-borne encephalitis virus, but also several other virus strains differing substantially from the agent of tick-borne encephalitis.

The first of these strains were isolated by the Czechoslovak group from a suspension of ground hungry *Ixodes persulcatus* females collected in the taiga forest in the neighbourhood of the village Romanovka, Kemerovo region, and from the spinal fluid of two patients hospitalized with a suspect febrile form of tick-borne encephalitis due to previous tick bites. Attention was attracted by the fact that these virus strains produced a complete cytopathic effect in chick embryo cell cultures within 48—72 hours, multiplied well upon inoculation into the yolk sac of 7-day-old chick embryos and caused a fatal illness after 2-5-days' incubation in newborn white mice but, as a rule, were not pathogenic for adult white mice. The use of hyperimmune diagnostic sera in neutralization tests in chick embryo cell cultures permitted to rule out any antigenic relationship of the new strains with arbor viruses of the tick-borne encephalitis, Western and Eastern equine encephalomyelitis and St. Louis and Japanese B encephalitis.

Soon after these first findings other laboratory groups of the expedition succeeded in isolating, in Kemerovo and Novokuznetsk, more than 20 similar

strains of the virus from *Ixodes persulcatus* ticks, and from the blood and spinal fluid of patients suspect of tick-borne encephalitis and of healthy persons bitten by ticks. These strains easily passed through Seitz asbestos pads.

Neutralization tests with some of these isolates performed at Moscow at the Institute of Poliomyelitis and Viral Encephalitides confirmed the antigenic differences of these viruses from the tick-borne encephalitis virus as well as from other types of Group A and B arbor viruses.

The new isolates are being investigated in detail in our laboratories parallelly at Moscow and Bratislava. So far, the following data to be published later in greater detail have been established.

The new isolates are markedly pathogenic on intracerebral inoculation, not only for newborn white mice but also for newborn white and cotton rats and newborn Syrian hamsters. Adult hamsters and adult white mice developed disease and died in occasional cases only. Intracerebral inoculation of *Macacus rhesus* monkeys resulted in short-lasting fever and viraemia. The new isolates caused a cytopathic effect not only in chick embryo and pig embryo kidney cell cultures, but also in human embryo fibroblast, primary and stable human amnion, HeLa, and primary monkey kidney cells. They formed big definite plaques in chick embryo cell cultures under agar overlay.

Neutralization tests with the new isolates done in chick embryo cell cultures with 93 sera from patients hospitalized with febrile disease after tick bite and possessing no detectable antibody against tick-borne encephalitis virus were definitely positive in 57 cases (61.3%). In a similar neutralization test with 21 paired sera from patients (taken in the first days of the febrile illness after tick bite and about 2 weeks later), a considerable increase of antibody titres in late sera was found in 10 cases, whereas in another 11 cases a high antibody titre was observed in both early and late serum specimens.

It could be suggested that the new virus isolated from *Ixodes persulcatus* ticks is the causative agent of some cases of human febrile disease.

Acta virol. 7 : 84—87, 1963

Multiplication Dynamics of Phase I and II *Coxiella burneti* in Different Cell Cultures

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Received July 23, 1962

Roberts and Downs (1959) studied the multiplication of *Coxiella burneti* (*C.b.*) in L mouse fibroblast and chick fibroblast cells. They found a decrease in the LD_{50} titre (on titration of tissue culture materials in chick embryos) by 4 log units two hours after inoculation of the cell cultures and suggested that this decrease is caused by the fact that many *C.b.* do not penetrate into the cells. They examined samples from the cell cultures taken 2, 24, 48 and 72 hours after infection. Using the fluorescent antibody method the authors mentioned observed intracellular *C.b.* as late as 21 hours after infection and reported that no *C. b.* were visible in the cells 5 hours after inoculation.

When studying *C.b.* multiplication in carcinomatous cells we found a decrease in the number of microscopically visible *C.b.* in the early stages of infection of the cell cultures. In phase contrast we did not observe binary fission of intracellular *C.b.* (Kordová and Kvíčala, 1962). Electron microscope study of ultrathin sections of Detroit 6 cells infected with *C.b.* showed that the mode of *C.b.* multiplication probably differs from that of bacteria (Rosenberg and Kordová, 1962).

The present paper reports investigations on the multiplication of *C.b.* in 3 different cell lines aimed at demonstrating the eclipse phase in early stages of infection. We used *C.b.* in two different phases in order to show whether there are any differences in this respect between fresh strains and strains passed for long periods in the yolk sac of chick embryo.

Materials and Methods

Strains of C. burneti: strains Geschwandtner and L_{35}, isolated from patients' blood during two Q-fever epidemics in Slovakia (Brezina, 1956), were used. Strain Geschwandtner was in phase II, strain L_{35} in phase I.

Cell strains. Detroit 6 (Berman and Stulberg, 1956), amnion (Fogh and Lund, 1957) and HEp-2 (Moore *et al.*, 1955) cells were used. They were grown by routine methods in the corresponding media with the addition of penicillin (200 units/ml.) and streptomycin (200 μg./ml.).

Infection of cell cultures. Fifty-ml.-bottles were seeded each with 2×10^6 cells in maintenance medium (Hanks' solution with 10% heated horse serum, 0.5% lactalbumin hydrolysate plus antibiotics as above) and incubated for 24 hours at 35.5° C. After withdrawal of the medium the cell cultures were inoculated with 2 ml. of 10^{-2} dilutions of 10% infective yolk sac suspensions in saline pH 7.2. Both *C.b.* strains reached in the yolk sacs titres varying from 4×10^9 to 4×10^{11} per ml. The inoculum was left in contact with the cells for 2 hours at 35.5° C. The cell cultures were then washed five times with Hanks' solution, supplied with maintenance medium and incubated at 35.5° C. At intervals of 0 (the end of the 2 hours' adsorption period), 6, 12, 24, 36 and 48 hours four cultures were pooled and after 5 cycles of freezing (on dry ice) and thawing (at 37° C) the materials were inoculated into the yolk sacs of 6 days old chick embryos (4 embryos per sample).

Infectivity titrations. Serial tenfold dilutions were inoculated in 0.25 ml. amounts into the yolk sacs of 6 days old chick embryos. Smears were examined microscopically and the ID_{50} values were calculated according to Reed and Muench (1938).

Results

As is evident from the results illustrated in Fig. 1, there occurred a decrease in the infectivity titres in the early stages of infection of cell cultures with *C.b.* (6 hours after infection). The decrease was marked in cells in which *C.b.*

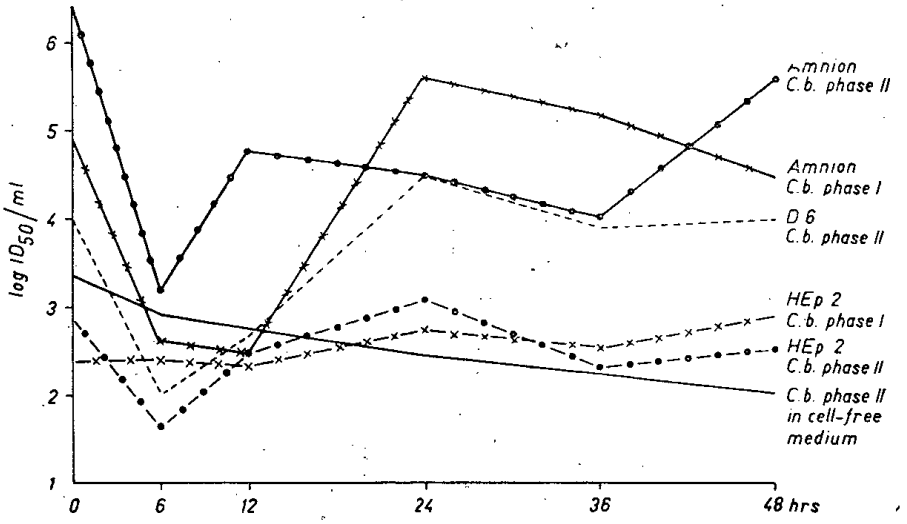

Fig. 1.
Dynamics of multiplication of *C. burneti* (phase I and II) in amnion, D6 and HEp 2 cell

multiplies well (see Fig. 2), whereas it was insignificant or none in cells in which *C.b.* only survives (during an observation period of 48 hours).

Later, 12—24 hours after infection, there occurred a marked increase in infectivity titres, which was followed by their less marked and slow secondary decrease.

The experiments demonstrated a different sensitivity of the cells used against infection with *C.b.* No significant differences between multiplication of the strains in different phases was observed.

We tested the decrease of *C.b.* infectivity in cell-free maintenance medium under condition equal to those in the experiments proper. The decrease of *C.b.* infectivity in the course of 48 hours was very slow (Fig. 1); after 6 hours the infectivity titre decreased by only 0.5 log unit.

Discussion

Electron microscope studies of ultrathin sections of cells from yolk sac tissue cultures and Detroit 6 cell cultures infected with *C.b.* prepared at

different intervals after inoculation we found that no *C.b.* could be detected in the cells during early stages of infection in spite of the high multiplicity of infection used. Changes of infected cells and numerous Coxiella-like particles were observed as late as 48 hours and later after inoculation. The morphological appearance of *C.b.* did not offer evidence of their binary fission (Rosenberg and Kordová, 1960, 1962). The marked decrease in *C.b.* infectivity 6 hours

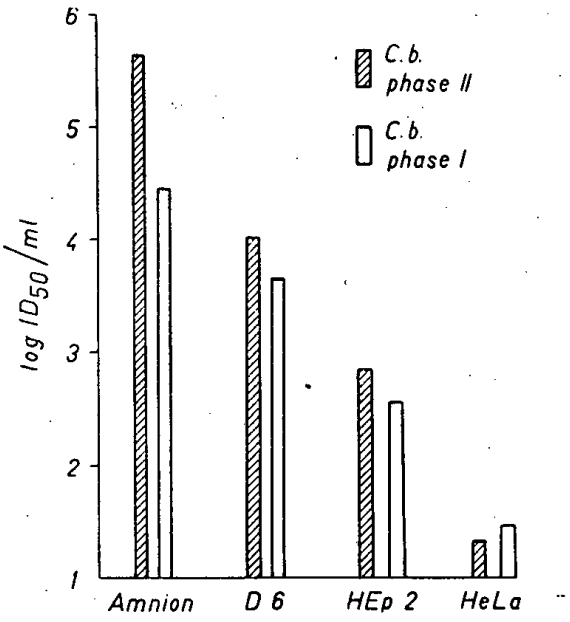

Fig. 2.

Multiplication of *C. burneti* (phase I and II) in different cell cultures 48 hours after inoculation

after infection of cell cultures is in no relation with the insignificant drop in infectivity occurring within the same period in a cell-free medium. We assume therefore that the drop in *C.b.* infectivity in early stages of infection can be interpreted as the eclipse phase.

Summary

Multiplication dynamics of *Coxiella burneti* in phase I and II were investigated in 3 different kinds of cells. A significant decrease in *C. burneti* infectivity during the early stages of infection was followed by a marked increase in infectivity. No differences in the multiplication dynamics were observed between *C. burneti* in phase I and II.

References

Berman, L., and Stulberg, C. S. (1956): Eight culture strains (Detroit) of human epithelial-like cells. *Proc. Soc. exp. Biol. (N.Y.)* **92,** 730.

Brezina, R. (1956): Experiences from the laboratory diagnostics of Q fever. I. *Bratisl. lek. Listy* **36,** 38 (in Slovak).

Fogh, J., and Lund, R. O. (1957): Continuous cultivation of epithelial cell strain (FL) from human amniotic membrane. *Proc. Soc. exp. Biol. (N.Y.)* **92**, 867.

Kordová, N., and Kvíčala, P. (1962): *Coxiella burneti* in tissue cultures studied by the optic microscope and in phase contrast. *Folia microbiol.* **7**, 89.

Moore, A. E., Sabachewsky, L., and Toolan, H. W. (1955): Cancer culture characteristics of four permanent lines of human cancer cells. *Cancer Res.* **15**, 598.

Reed, L. J., and Muench, H. (1938): A simple method of estimating fifty percent endpoints. *Amer. J. Hyg.* **27**, 493.

Roberts, A. N., and Downs, C. M. (1959): Study of the growth of *C. burneti* in the L strain mouse fibroblast and the chick fibroblast. *J. Bact.* **77**, 194.

Rosenberg, M., and Kordová, N. (1960): Study of intracellular forms of *Coxiella burneti* in the electron microscope. *Acta virol.* **4**, 52.

Rosenberg, M., and Kordová, N. (1962): Multiplication of *Coxiella burneti* in Detroit 6 cell cultures. An electron microscope study. *Acta virol.* **6**, 176.

Acta virol. 7 : 88—89, 1963

Effect of Some Inhibitor-Destroying Substances on the Nonspecific Inhibitor of C Influenza Virus Present in Normal Rat Serum

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Received June 23, 1962

As reported previously (Styk, 1954), serum from white rats contains in considerable titres a substance which inhibits haemagglutination by C influenza virus. In further papers (Styk, 1955; Hána and Styk, 1959, 1960) we have described some properties of this nonspecific, though considerably selective (acting almost against C influenza virus only) inhibitor, named C-inhibitor. The present paper reports further data on the nature of C-inhibitor and on its sensitivity to some frequently used inhibitor-destroying substances.

The C/Czechoslovakia 1/52 influenza virus strain isolated by us in 1952 was used in the form of infective allantoic fluid. Ten-day-old chick embryos were inoculated into the allantoic cavity with 10^2—10^4 EID_{50} of virus. The allantoic fluid was harvested after 72—84 hours' incubation at 32—33° C, which temperature is suitable for a satisfactory multiplication of C influenza virus.

Haemagglutination-inhibition tests were done by the micromethod of Takátsy (1955). Normal rat sera were examined individually, either fresh or after storage for several weeks at —20° C. The methods used for treating the sera with inhibitor-destroying substances have been described previously (Styk and Hána, 1961).

The effects of the different serum treatments on the activity of employed C-inhibitor are evident from Tables 1 and 2. The titre of C-inhibitor remained substantially unaffected by treatment with potassium metaperiodate, receptor destroying enzyme (RDE), crude *Vibrio cholerae* filtrate or carbon dioxide. Only a 1% trypsin (Difco trypsin 1 : 250) solution caused a 16-fold or higher decrease in the titre of C-inhibitor. In addition, Borecký (1958) reported that his pneumococcal factor also caused a decrease in the activity of C-inhibitor.

Table 1. Effects of trypsin, RDE and crude V. cholerae filtrate on the titre of C-inhibitor in two normal rat sera heated at 56° C for 30 minutes

Treatment of serum	Serum No. 1	Serum No. 2
1% trypsin (37° C/30 mins, then 56° C/30 mins)	< 20	160
0.5% trypsin (37° C/30 mins, then 56° C/30 mins)	±40	1280
Crude *V. cholerae* filtrate (37° C/60 mins, then 56° C/30 mins)	160	2560
RDE (512 units) (37° C/60 mins, then 56° C/30 mins)	±160	1280
RDE (128 units). (37° C/60 mins, then 56° C/30 mins)	160	±5120
Untreated control	±320	2560

The results reported indicate that C-inhibitor is characterized by a different sensitivity to inhibitor-destroying substances as compared with type alpha, beta and gamma nonspecific inhibitors of myxoviruses, and that therefore it represents a substance differing from these inhibitors.

Table 2. Effects of potassium periodate and carbon dioxide on the titre of C-inhibitor in two unheated and heated rat sera

Treatment of serum	Serum No. 1	Serum No. 3
Unheated, untreated	320	±640
Unheated, KIO_4 M/50* (15 mins/22° C)	320	1280
Unheated, KIO_4 M/200* (15 mins/22° C)	320	320
Unheated, CO_2	320	±640
Heated (56° C/30 mins), untreated	640	640
Heated (56° C/30 mins), KIO_4 M/50*	±1280	±1280
Heated (56° C/30 mins), KIO_4 M/200*	640	640
Heated (56° C/30 mins), CO_2	±1280	640

* 1 part serum + 3 parts KIO_4 solution.

Heating of rat serum occasionally resulted in a moderate increase in the titre of C-inhibitor. A similar phenomenon is known to occur with type alpha and gamma inhibitors. The comparatively low titres of C-inhibitor can be explained by the use of the micromethod which yields lower titres than the test-tube method.

Summary

Data are reported on the sensitivity to some inhibitor-destroying substances of the nonspecific inhibitor of C influenza virus, occurring in normal rat serum. C-inhibitor proved to be sensitive only against the action of trypsin, which caused a 16-fold or higher decrease in the titre of C-inhibitor. KIO_4, RDE, crude *Vibrio cholerae* filtrate or CO_2 did not substantially affect the titre of C-inhibitor. These data supplement previous findings on the nature of C-inhibitor and offer additional evidence that this inhibitor differs substantially from the known type alpha, beta and gamma nonspecific viral inhibitors.

References

Borecký, L. (1958): A factor destroying virus receptors in pneumococcal cultures. II. The effect of "Pneumococcal factor 103" on erythrocyte receptors and inhibitors. *Acta virol.* **2**, 201—207.

Hána, L., and Styk, B. (1959): Some properties of influenza C virus haemagglutination inhibitor from rat serum. *Acta virol.* **3** (Supplem.), 85—90.

Hána, L., and Styk, B. (1960): Influence of delipidisation on the haemagglutination inhibiting activity of rat serum against influenza type C viruses. *Acta virol.* **4**, 392—393.

Styk, B. (1954): An epidemic outbreak of influenza caused by type C influenza virus. *Čsl. Hyg. Epid. Mikrobiol.* **3**, 137—145 (in Slovak).

Styk, B. (1955): Non-specific inhibitors in normal rat serum for the influenza C type virus. *Folia biol.* **1**, 207—213.

Styk, B., and Hána, L. (1961): Cofactor and specific antibodies against influenza viruses. II. The effect of substances destroying nonspecific inhibitors on the cofactor and on specific A2 antibodies. *Acta virol.* **5**, 342—350.

Takátsy, G. (1955): The use of spiral loops in serological and virological micro-methods. *Acta microbiol. Acad. Sci. hung.* **3**, 191—202.

Acta virol. **7**: 90, 1963 *Letter to the Editor*

COMPLEMENT-FIXING COXIELLA BURNETI ANTIGEN PREPARED FROM INFECTIVE YOLK SACS BY TRYPSIN TREATMENT

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Received August 22, 1962

The preparation of *Coxiella burneti* antigen by extraction with ether (*1*) yields a slightly infectious product, whose antigen titre in the complement-fixation reaction (CFR) depends on the loss of *C. burneti* in the middle layer consisting of debris and on the homogeneous distribution or aggregation of the Coxiellae. By means of the purification with trypsin described below the loss of antigenic material occurring on ether extraction is avoided and an optimal distribution of the Coxiellae in the antigen is obtained. Purification of *C. burneti* and psittacosis virus (ornithosis) with trypsin without loss of antigenicity has been mentioned by Haas (*2*).

We are not aware of any description of a practical method for the preparation of Q-fever antigen required for the CFR based on this observation in place of the ether extraction method.

Yolk sac material containing *C. burneti* of the strain Grita was triturated with sand, suspended in physiological saline (2 ml. per yolk sac) and the sand and tissue material were removed by centrifugation at 1000 rev/min. for 10 minutes.

The suspension containing the Coxiellae was centrifuged at 4500 rev/min. for 1 hr. and the sediment, free from the soluble portions of the egg and containing the Coxiellae, was resuspended in saline, 3 ml. per yolk sac.

One part of a 1% solution of "Trypsin powder" (Merck) in physiological saline clarified by centrifugation, was mixed with 3 parts of the mechanically partially purified suspension of Coxiellae. The pH of the mixture was adjusted to 8.0 and chloroform was added. Digestion proceeded at 42° C for 48 hrs, with controlling the pH after 4 hrs and occassional shaking. Chloroform and debris were then removed by centrifugation at 1000 rev/min. for 5 mins. Centrifugation for 10 mins at 2000 rev/min. was repeated once or twice. The supernatant fluid thus obtained was centrifuged at 4500 rev/min. for 1 hr. The sediment was washed twice with phosphate buffer. The final sediment was resuspended in 1 ml. per yolk sac of phosphate buffered saline and stored in a deep freeze without the addition of any preservative.

The Coxiellae thus obtained did not multiply either in animals or in eggs. The antigen titre in the CFR of this preparation was usually approximately 4 to 6 times that of the antigen obtained by the ether extraction, due to the homogeneous distribution of the Coxiellae and to the effect of trypsin in preventing the loss of antigen occurring on ether extraction.

Rabbits were immunised with 8 to 10 injections of this preparation. The specific antibody titres were similar to those obtained by using ether antigen. However, the trypsinised antigen showed by comparison with the ether antigen few nonspecific reactions with chicken egg proteins.

The experimental details will be published elsewhere.

References

1. Bengtson, I., *Publ. Hlth Rep (Wash.)* **59** : 402, 1944.
2. Haas, R., *Z. Hyg. Infekt.-Kr.* **135** : 566, 1952.

Acta virol. **7** : 91, 1963

Letter to the Editor

REMOVAL OF THERMOSTABLE INHIBITORS AGAINST A2 INFLUENZA VIRUS FROM IMMUNE HORSE SERA BY RIVANOL

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Received September 8, 1962

Diagnostic anti-influenza sera produced in the U.S.S.R. by hyperimmunization of horses-producers proved unsuitable for serological identification of A2 influenza virus strains which became prevalent since 1957. This was caused by a regular occurrence in all sera from normal and immunized horses of previously unknown thermostable inhibitors neutralizing, in high titres the so-called inhibitor-sensitive A2 influenza virus strains both in the haemagglutination-inhibition (HI) test and virus neutralization test done in developing chick embryos or suspended chorionallantoic membrane tissue cultures. Similar inhibitors against A2 influenza virus occur in high titres in guinea pig, rabbit and white rat sera. According to their behaviour regarding these inhibitors, which have been shown biochemically to be alpha-2-globulins (*1*, *2*), A2 influenza virus strains can be readily divided into inhibitor-resistant and inhibitor-sensitive strains.

Treatment of sera with receptor destroying enzyme or their saturation with CO_2, destroying inhibitors against A, A1 and B influenza virus, did not affect the tehermostable inhibitors against A2 virus strains. Treatment of sera with potassium periodate removed the thermostable inhibitors against A2 virus strains, but left unaffected those against A, A1 and B viruses, and, in addition, caused a marked decrease in the titre of specific antibodies.

To simultaneously remove from horse serum the thermostable inhibitors against all serological variants of types A and B influenza virus and completely preserve the specific antibodies, we successfully employed rivanol treatment of the sera, which has become widely used for the isolation of gamma-globulin (*3*).

In our experiments we used individual sera prepared by hyperimmunization of horses with type A, A1, A2, B and C influenza and Sendai parainfluenza viruses, respectively. To remove the inhibitors, 4 volumes of a freshly prepared 0.4% rivanol solution pH 7.0—7.3 were added to 1 volume of native serum. After mixing for 20 minutes in the cold, the precipitated proteins were separated by centrifugation at 2000 rev/min. for 10 minutes at +2° C. The supernatant fluid thus obtained, which contianed the principal part of specific antibodies, was freed of excess rivanol by activated charcoal. Then each sample was titrated in parallel with the original serum in HI tests with all serotypes of influenza virus mentioned above. Several A2 influenza virus strains, differing in their sensitivity to antibodies and inhibitors, were used.

The results showed that it was possible by rivanol treatment to remove from horse seum all or almost all inhibitors against the different types of influenza virus, including those against A2 influenza virus. The original titre of inhibitors against A2 influenza virus in heated sera was from 1 : 5000—1 : 20 000; after rivanol treatment their titre decreased to 1 : 5—1 : 40. The titres against A, A1 and B influenza viruses decreased respectively from 1 : 160—1 : 20 to 1 : 10 or less, and of those against Sendai parainfluenza virus from 1 : 80—1 : 320 to 1 : 20 or less. At the same time the titres of homologous antibodies in the horse sera decreased maximally 2—4 times, i.e. their level remained high making thus possible the identification of recently isolated influenza virus strains belonging to any serotype with dilutions of 1 : 40 or higher. At such dilutions the inhibitors were absent and the results of HI tests were completely specific.

References

1. Hána, L., Styk, B., and Kočišková, D., *Acta virol.* **4** : 356, 1960.
2. Polyak, R. Ya., and Smorodintsev, A. A., *Acta virol.* **5** : 1, 1961.
3. Hořejší, J., and Smetana, R., *Acta med. scand.* **155** : 65, 1956.

VIIIth International Congress for Microbiology, Montreal, August 19 — 24, 1962

On this international gathering of workers in all fields of microbiology it was possible to observe a greater interest paid to virological problems than on the last Congress held four years ago in Stockholm. The present report will give a short summary of the virological items dealt with at Montreal.

The Organization Committee decided upon 3 forms of presenting papers dealing with the individual selected topics: symposia, panel discussions and focal topic sessions. Virological problems were dealt with in symposia and focal topic sessions. In addition to these public meetings, members of the Subcommittee on Viruses met before and during the Congress to discuss especially problems concerning the nomenclature of viruses.

There were 2 symposia devoted entirely to virological problems: (1) interference and interferon and (2) demonstration of viruses in tumours. In the symposium on mechanisms of immunity one of three papers presented dealt with immunity mechanisms in viral infections.

The symposium on interference and interferon was presided over by A. W. Downie (Liverpool). A. Isaacs (London) presented an introductory lecture on the basic principles and modes of action of interferon. He gave a definition of the properties of interferon which is formed intracellularly as a reaction of the cell on virus penetrated into it, and which inhibits the formation of viral nucleic acid or blocks its synthesis completely.

Interferon thus represents a factor participating in the process of recovery of the host from a viral infection, or inducing a chronic viral infection. But in the process of recovery interferon is not the only effective factor, in addition to the so-called natural resistance factors the most important ones actively participating in the course and affecting the result of viral infections are the temperature, increased oxygen tension in the environment and the quality of cell systems. Interesting is the relation between the virulence of viruses and their ability to induce interferon formation. Avirulent strains of measles virus produce more interferon than virulent ones.

The first experimental results concerning the effect of interferon in man were demonstrated on a reduction of local lesions caused by inoculation with vaccinia virus. Further research is needed to show how, when and why interferon is formed following the contact of different cells with virus, and which is the biological function of interferon formation.

The basis of the paper by D. Blaškovič and J. Vilček (Bratislava) was a study of the interference phenomenon and interferon formation in infections caused by arborviruses. Interferon obtained from chick embryo cells infected with tick-borne encephalitis virus has properties similar to those of interferon from chorioallantoic membranes inoculated with live or inactivated influenza virus. So far, however, it was impossible to obtain interferon from cell systems inoculated with inacitvated tick-borne encephalitis virus.

J. P. Fox (New York) reported about the epidemiological significance of interference phenomena which result in blocking a second viral infection, if the latter occurred during the development of the preceding viral infection. It is very important to follow and accurately interpret these phenomena, especially after introduction of the use of live vaccines (with 17 D virus, E strain of spotted fever and attenuated strains of polio and measles viruses).

The symposium on the demonstration of viruses in tumours was presided over by A. B. Sabin (Cincinnati). F. L. Horsfall (New York) reported about the role of infectious agents in tumour pathogenesis, W. Bernhard (Villejuif) about recent findings obtained by electron microscopy of cancerogenous viruses, and G. Klein (Stockholm) about genetic aspects of viruses and tumours.

The discovery of viruses capable of inciting in animals the formation of tumours clinically and histologically resembling some human tumours has been followed by an increasing interest in research into these problems. At present, investigations are being carried out on a number of models: Rous sarcoma, polyoma virus, SV 40, Ehrlich's carcinoma and blood tumours of rodents. Further viruses have been recently obtained from animals. Those occurring in monkey tissues used for the preparation of vaccines for human use have been the subject of extensive discussions resulting in measures introduced with the aim of preventing the introduction of these viruses into the human population. Although the work on tumour viruses has several features in common with that on animal and plant viruses, one must be very cautious in generalizing the results

or in attempting to apply them directly in human pathology. The problem of viral etiology of tumours, though experimentally demonstrated as yet in animals only, is, however, extremely important and deserves a concentrated study.

J. E. Salk (Pittsburgh) demonstrated on his own material gained in studies on influenza and poliomyelitis viruses how immunity processes are induced and how they persist in man with respect to viruses used in inactivated vaccines.

The following virological items were dealt with in the focal topic sessions: the structure of viruses, viruses and tumours, and respiratory viruses (Aug. 20); biochemistry of viruses and recently isolated viruses (Aug. 21), and genetics of viruses and viral and rickettsial zoonoses (Aug. 23). In works presented in other sections viruses, including bacteriophages, were used as models (metabolic control of cellular synthesis, structure and function of the cytoplasm). Thus it is evident that specialization made a considerable progress also in virology and that virologists interested in different general and special problems often had difficulties in deciding in which session to participate. It is impossible, therefore, to present a detailed account of all the papers presented. I will only mention those problems to which recently the greatest attention has been paid, naturally with respect to the reports presented and not with respect to the interests of all virologists. The comments, which follow, are influenced by my personal opinions about the importance of some problems and the omission of others does not mean that they are less important.

The structure of viruses was the subject of only a few papers. The studies involved morphology in the electron microcope, antigenic composition of the whole virus and the process of infection of the cell.

Investigations were reported on biological properties of some animal tumour viruses and the isolation was announced of viruses which could have some relations to human tumours (sarcoma, carcinoma), but their etiological role was not considered definitely proved.

Papers on respiratory viruses dealt with their structure, properties of their subunits, and their abilities to induce interferon formation and react with nonspecific inhibitors (influenza viruses). Major interest has been paid to the latter two problems. The epidemiological importance of myxoviruses was repeatedly stressed.

Biochemical studies on viruses involved the properties of viral nucleic acids, their synthesis and the effects of certain antimetabolites (5-bromedeoxyuridine and interferon, to which this property is ascribed).

In the section on recently isolated viruses, the greatest interest was paid to further reports on the properties of the hepatitic virus isolated in Parke and Davies laboratories in Detroit. The request was put forward in the discussion that reports on the properties and quality of new viruses should be considered valid only if making available the respective virus strains to other virus laboratories. A more accurate classification of the individual groups within arborviruses has been attempted according to the antigenic properties of viruses. The isolation of an as yet unidentified virus from a case of chronic follicular conjunctivitis was announced.

Genetic studies were done mostly on animal viruses. Phages were the subject of only one paper. The other reports dealt with the properties of variants and mutants of Coxsackie 9, polio, Newcastle disease and ECHO 9 viruses and of myxoviruses. The character of the works was no more only descriptive; the mechanisms and causes of the origin of mutants and variants were also investigated.

The section of viral and rickettsial zoonoses contained reports on the biological properties of rabies virus, reovirus, foot-and-mouth disease virus, Teschen disease virus and arborviruses, including the formation of interferon by arborviruses and the use of inactivated virus for immunization.

The short survey of the virological activities on the VIIIth International Congress for Micro biology may be concluded in stating that the activities of virologists are continuing to increase as evidenced also by a remark by one of the virologists, who divides the organisms into the viruses and the others. The prevailing interest has been moving recently to the genetics, biochemistry and structure of viruses and to tumour viruses. In all these fields considerable progress can be expected which will add to our knowledge of the pathogenesis of viral infections and of the viral etiology of tumours.

The Congress was closed by a general meeting of the International Association of Microbiological Societies, on which a report about the activities after the last Congress in Stockholm, 1958, was presented. André Lwoff (France) was elected president, C. G. Hedén (Sweden) vice-

president, N. E. Gibbons (Canada) secretary general, M. Welsch (Belgium) treasurer and V. D. Timakov (Moscow) member of the Association's council. Soviet colleagues promised to clear up the possibilities of organizing the next congress in 1966 in Moscow.

The sessions of the present Congress took place in Queen Elizabeth Hotel fulfilling all spatial and technical requirements of such an enterprise. The number of participants registered until August 22 amonnted to 2013. The course of the Congress was smooth and the scientific part agreeably overlapped with the social programme. The Canadian organizers and hosts, and the whole Organization Commitee, must be congratulated to their work. The result of their efforts, which are deeply appreciated, was that we felt very well at the Congress and brought back to our laboratories agreeable memories and many stimuli for further work.

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A Jubilee of Czechoslovak Science

Ten years ago the leading scientific institutions existing at that time in Czechoslovakia were reorganized to give rise to the Czechoslovak Academy of Sciences. The new Academy started from the best traditions of Czech and Slovak science (The Czech Academy of Sciences and Arts, The Royal Czech Society of Sciences etc.) and during the past 10 years which elapsed since its foundation it played a role of primary importance in planning and in the manysided development of scientific research in Czechoslovakia, which has been warmly appreciated by Czechoslovak scientists.

The Institute of Virology of the Czechoslovak Academy of Sciences at Bratislava was founded on January 1st, 1953, a few weeks after the Academy itself has come into existence. When mentioning this event in Acta virologica we are not doing so because we consider 10 years a period sufficient for a precise evaluation of the scientific work done by this Institute and for deciding whether its existence was justified or not. This task will remain a hard one also in the future, because even in the era of cosmic flights, as Prof. J. D. Bernal says, "biology still remains a chaotic subject". In the case of an institute devoted to virus research another factor must be added, namely the general youth of this discipline. But we are meeting the 10th anniversary with feelings of a healthy optimism. Ten years ago there was neither in Czechoslovakia nor elsewhere in Europe an institute of general virology which could have served us as a complete, ready example. The concept of Academician D. Blaškovič, the founder and director of the Institute of Virology, was a broad virological approach to research on viruses and viral diseases, supported by an extensive application of recent advances in biochemistry and other bordering disciplines. There was no understanding for such a concept before the liberation of Czechoslovakia after World War II and favourable conditions were created only after the establishment of the Czechoslovak Academy of Sciences. In 1953 we started with 16 workers possessing university training, but only a few of them an experience in virology. During the first ten years of its existence the Institute of Virology fulfilled two tasks with the same intensity: it formed its research programme and trained research workers for its own needs and for other research or diagnostic institutions in Czechoslovakia.

The growth of the Institute of Virology can be illustrated for example by the increase in the number of publications. Whereas in 1953 sixteen workers published 6 original papers, five years later in 1958 thirty-six workers published 63 papers, and after 9 years, in 1962, fourty workers published 113 original experimental papers.

Lively scientific correspondence with virologists all over the world offers evidence of the echo of these publications. Exchange of experiences has also been effected by personal contacts. From 70—100 foreign investigators visit the Institute each year and their remarks in the guest book indicate that they are leaving us with friendly feelings. The endeavour of initiating and maintaining friendly scientific contacts with virologists of different nations is a characteristic feature of the staff of the Institute of Virology. Such was also the spirit prevailing on the conferences and symposia organized by the Institute during the past ten years, in which participated over 120 virologists from abroad in addition to domestic workers.

We assume that a good work has also been done by the journal Acta virologica, whose editoria

office is located at the Institute of Virology. Since its foundation in 1957 it has spread even to remote virological laboratories throughout the world, informing about the investigations done at the Institute of Virology itself and at other laboratories in Socialist countries.

The Institute of Virology comprises 4 research departments dealing respectively with respiratory, neurotropic and plant viruses, and rickettsiae. The principal models worked with are the myxoviruses, tick-borne encephalitis virus, Coxiella burneti and leafhopper-borne plant viruses. Chief interest has been paid to pathogenesis, immunity factors, and also to ecology of these infections and to the structure of their causative agents. For details the reader is referred to the respective chapters in the book "Advances in Biological Sciences", published recently by the Czechoslovak Academy of Sciences.

Armed with the experiences gained during the past 10 years (which Academician Blaškovič calls the "elementary school" period) we are entering the second decade with undiminished endeavour to contribute actively and with increasing efforts to the elucidation or the "riddle of viruses". We are convinced that it will be possible to fulfil these aims because of several reasons, of which a rather important one is the fact that the average age of the scientific staff members is low (35) and that they are thus capable of further development. To add, the Institute is headed by a director who reached the age of the fullest expansion of his creative energy. It is by chance that the year of the10th anniversary of the Institute of Virology is also that of the 50th anniversary of its director Academician D. Blaškovič, but the connection between the characteristic features of the Institute and the personal and scientific profile of its director is not. On the occasion of this double jubilee the staff members of the Institute of Virology sincerely appreciate the work done by their director, advisor and friend.

We wish ardently that the Institute of Virology of the Czechoslovak Academy of Sciences be stepping forward in the next, riper decades with an enthusiasm which was characteristic of the period from 1953 to 1963.

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Viruses

K. M. Smith; Cambridge, at the University Press, 1962; 134 pages; price 21 s.

Within a relatively short interval this is a second book on viruses published by the Cambridge University Press. Whereas the first one (Waterson: Introduction to animal virology) has been written for persons possessing a background in biological or medical sciences, the present publication is destined for a much broader range of readers. Any virologist will be familiar with almost all the items dealt with by K. M. Smith in a very understandable manner, but reading of the book will offer many new ideas and stimuli for further work.

In addition to the preface, a list of pertinent literature and a subject index, the book is divided into 12 chapters. After having discussed the meaning of the term "virus" and after a brief historical introduction Dr. Smith presents selected data on various viral diseases of man, animals, plants, arthropods, protozoa and bacteria, but in accordance with the general arrangement of the book he gives no clinical descriptions of these diseases. Nor can much information on the latter point be obtained from the illustrations presented, but this can be hardly considered as a drawback. As next, six pages are devoted to the isolation of viruses, their chemical nature and virus crystals, but twice as much space to electron microscopy of viruses and its applications. A somewhat similar situation can be encountered in the case of chapters 6 and 7, dealing with the spread of viruses and virus vectors, respectively. But this disproportion is only natural if the importance of electron microscopy in the study of viruses or that of vectors in the spread of viruses should not be underestimated. Separate chapters are devoted to multiplication of viruses (including some data on tissue cultures), viruses affecting invertebrate animals, latent virus infections, tumour viruses, control of viruses and viruses as agents of biological control.

Almost any author of a publication of this kind in any field of science will base the selection of material on his personal experience. For those acquainted with the pioneering work of K. M. Smith on plant and insect viruses it will be not too hard a task to find out that this fact became

manifested also in the selection of facts and opinions in his new book. Some specialists in other fields of virology would perhaps prefer to include additional examples, reduce or extend these or other parts, or to explain some phenomena from different aspects, but the "Viruses" will certainly fulfil the principal aim of disseminating knowledge of viruses among those wishing to obtain a general information about these pathogens.

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ERRATUM

Vol. **6**, p. 524 (paper by Szántó et al.), first sentence: *for* "It has been shown that alpha-inhibitor of influenza virus splits off sialic acid from the virus so that the haemagglutination . . ." *read* "It has been shown that virus splits off sialic acid from the alpha-inhibitor of influenza virus so that the haemagglutination . . "

Acta virologica — an international journal, published under the auspices of the Czechoslovak Academy of Sciences in the Publishing House of the Czechoslovak Academy of Sciences (Vodičkova 40, Praha 1-Nové Města, dod. pošt. úřad 1). Adress of the editor: Institute of Virology of the Czechoslovak Academy of Sciences, Mlýnská dolina, Bratislava 9. Printed by Knihtisk, n. p., závod 4 (Sámova 12, Praha 10-Vršovice, dod. pošt. úřad 101). 6 issues a year. Annual subscription Kčs 66,—, US $15,40, £5.10.0. Single copies Kčs 11,—, US $2,60, £0.18.4. Subscription inquiries from abroad, except from socialist countries, may be sent to ACADEMIC PRESS Inc. (London) Limited, Berkeley Square House, Berkeley Square, London, W. 1, or to ACADEMIC PRESS Inc., Publishers, 111 Fifth Avenue, New York 3, N. Y., or to Artia, Ve smečkách 30, Praha 1-Nové Město, P. O. Box 790, Czechoslovakia.

This number has been issued in january 1963.

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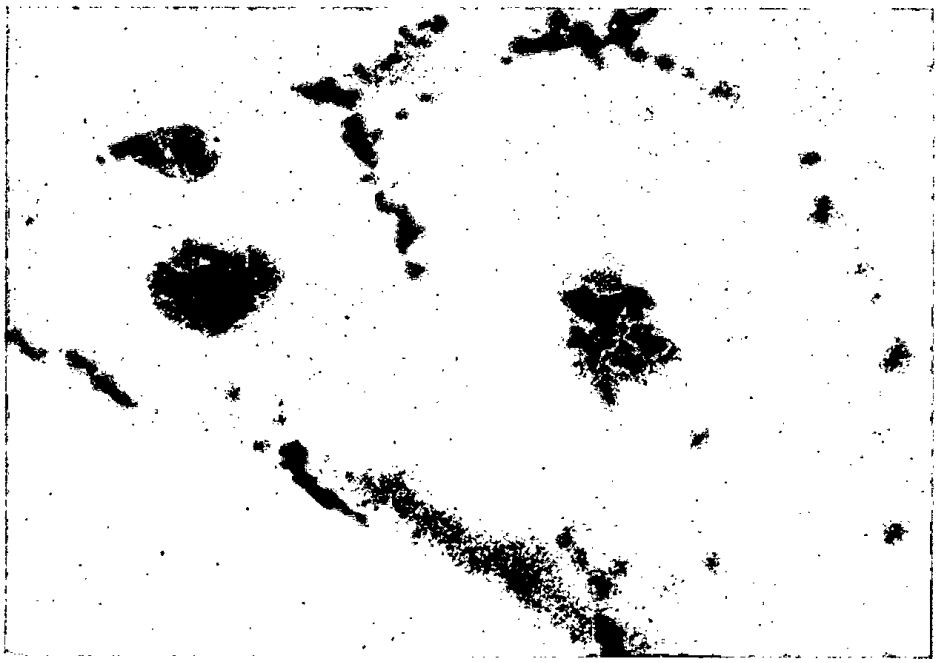

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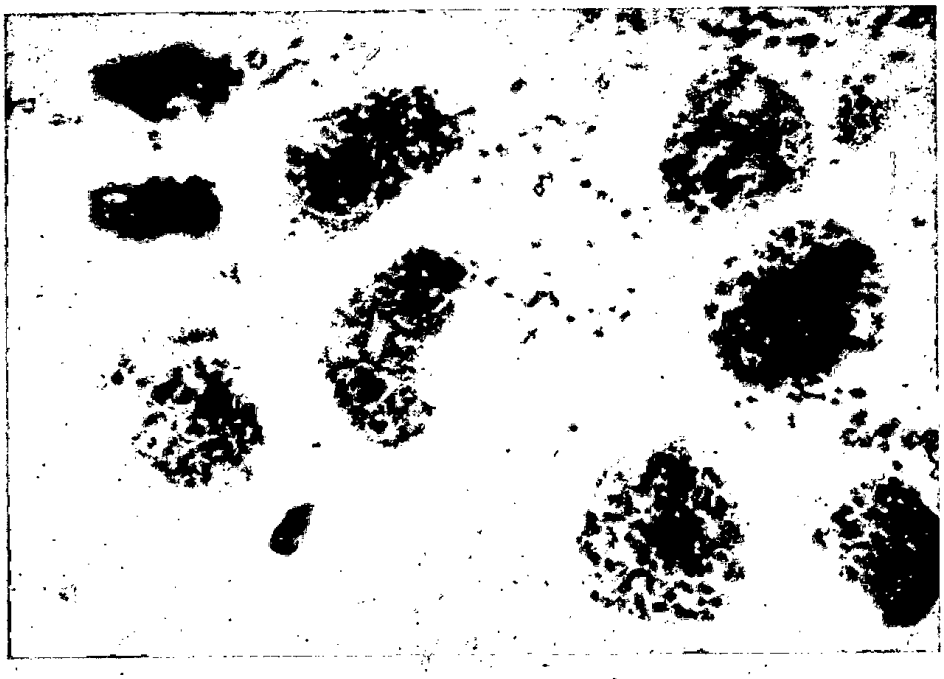

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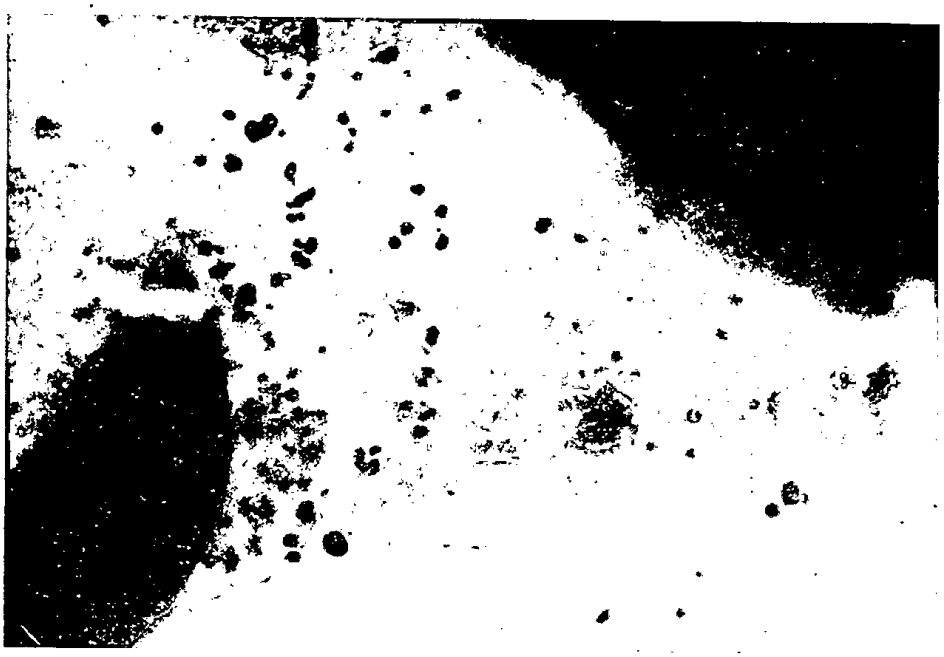

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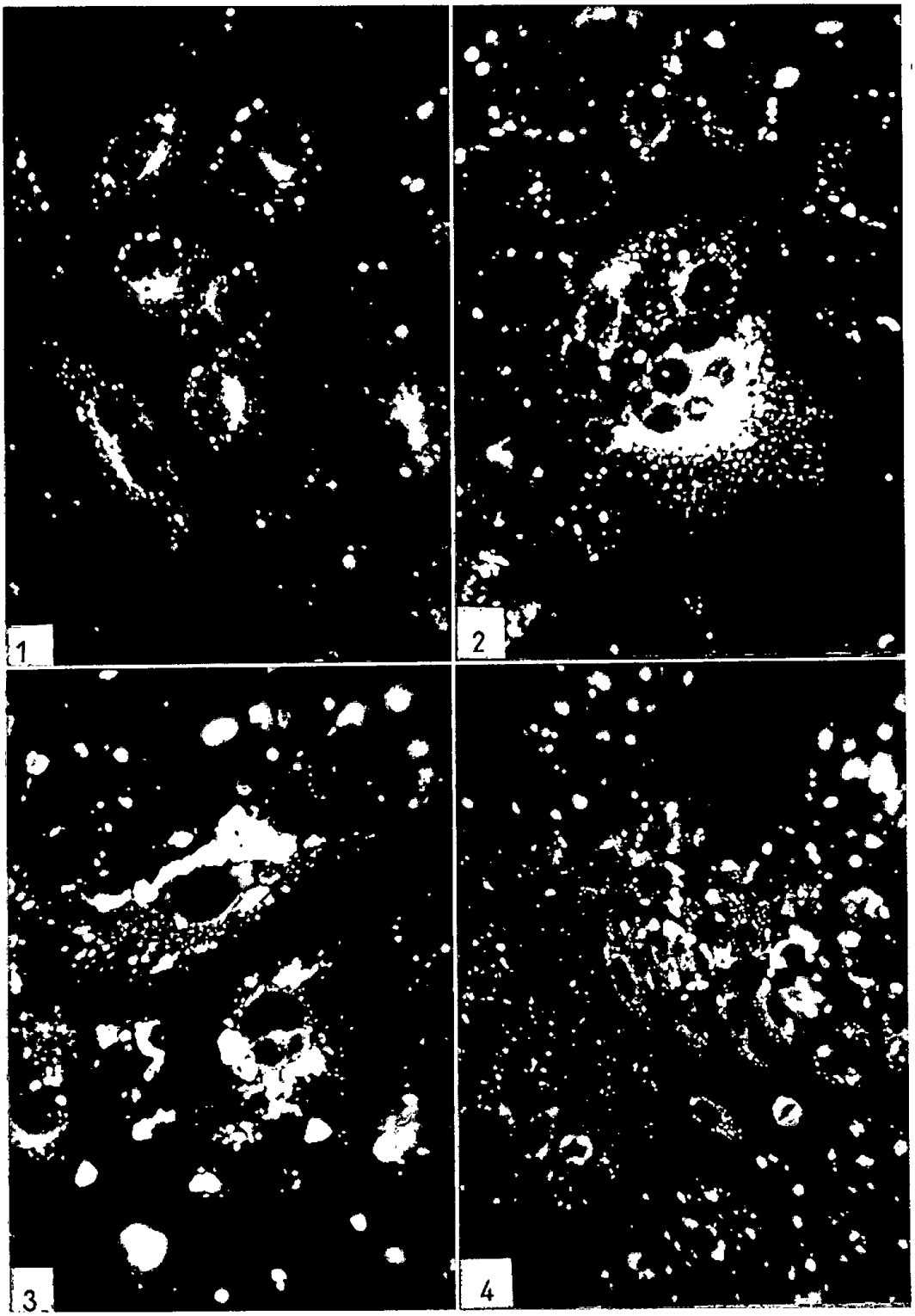

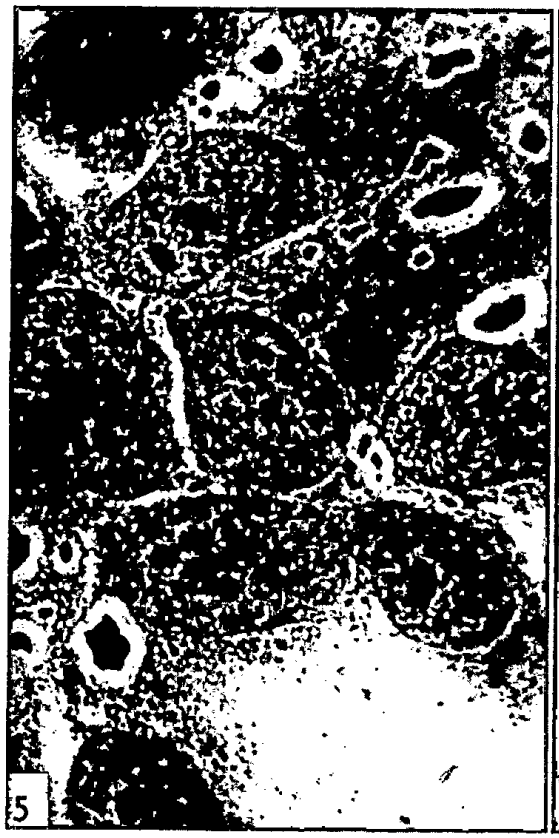

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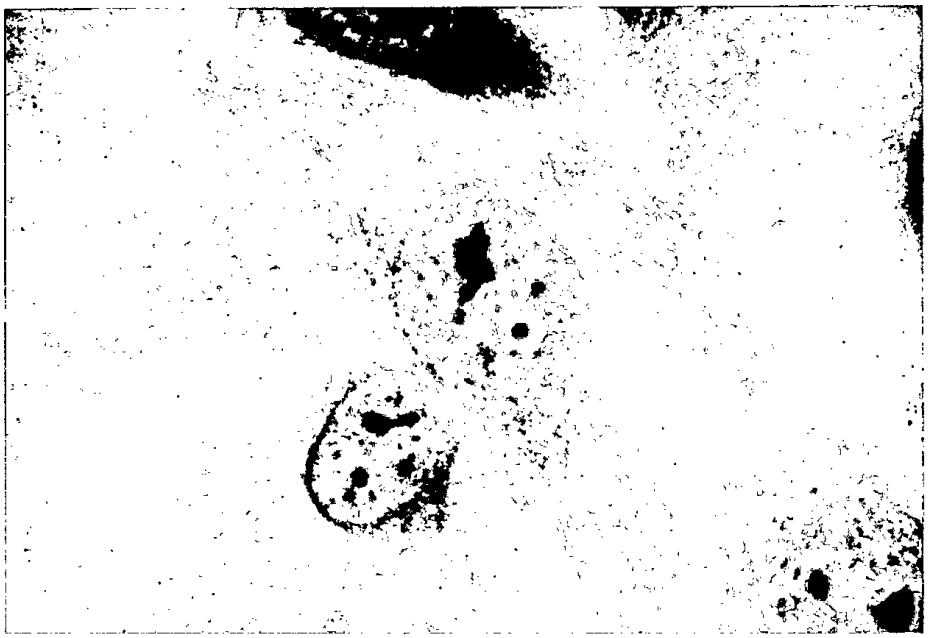

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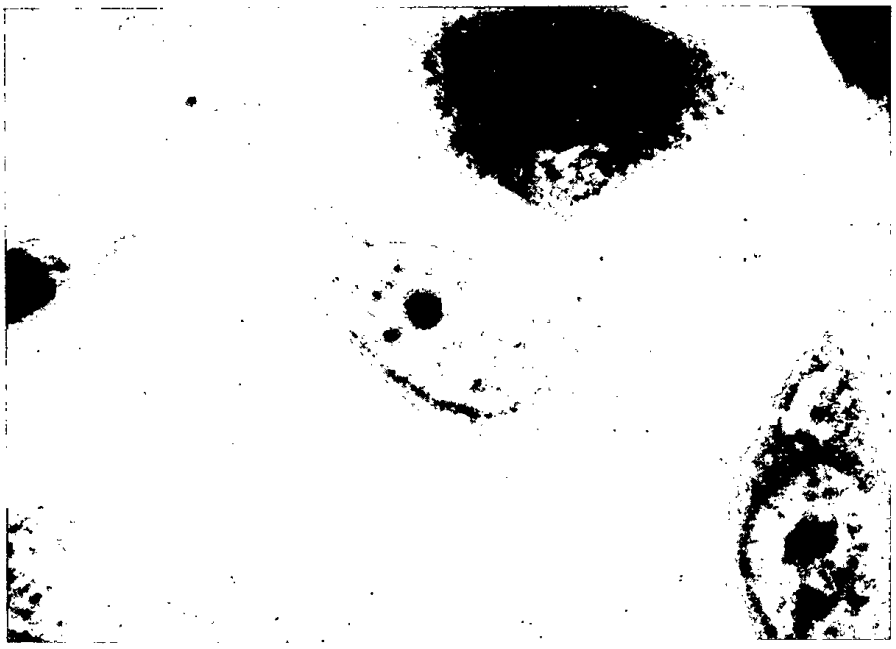

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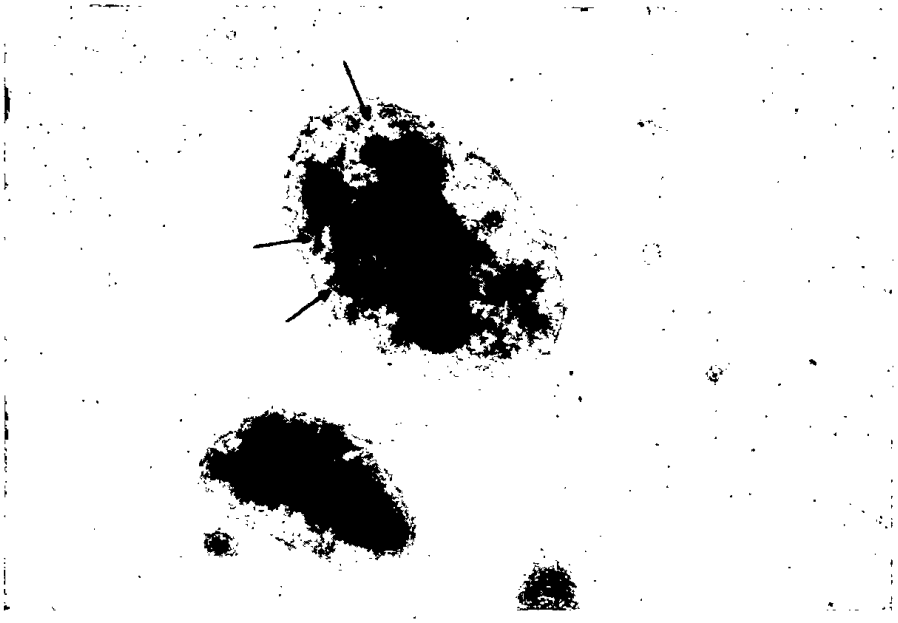

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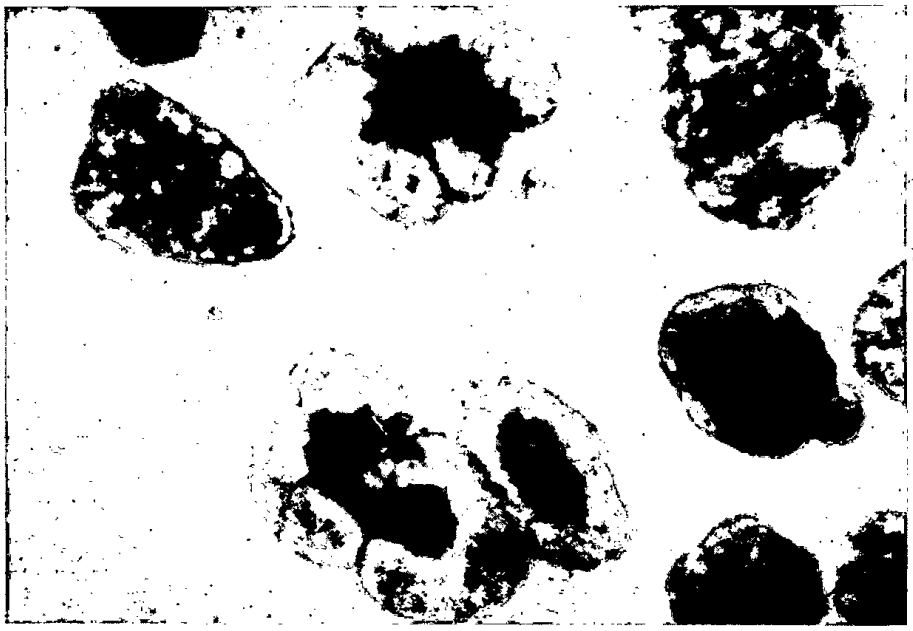

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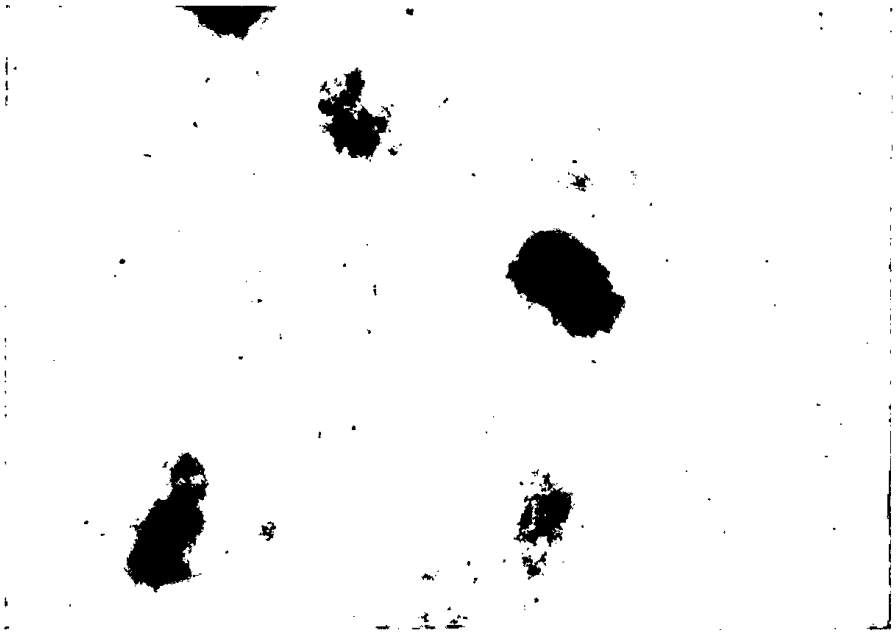
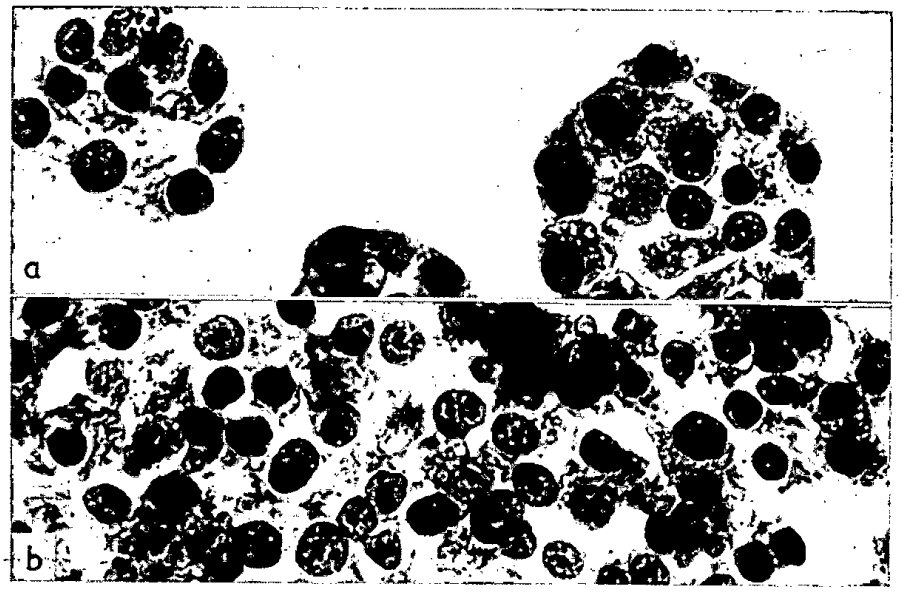


Fig. 5.

Fig. 6.

Zalkind, S. Ya., Andzhaparidze, O. G., Bogomolova, N. N., Fokina, A. M.: Morphological and Cytochemical Study of HEp-2 Cell Cultures Persistently Infected with Tick-borne Encephalitis Virus



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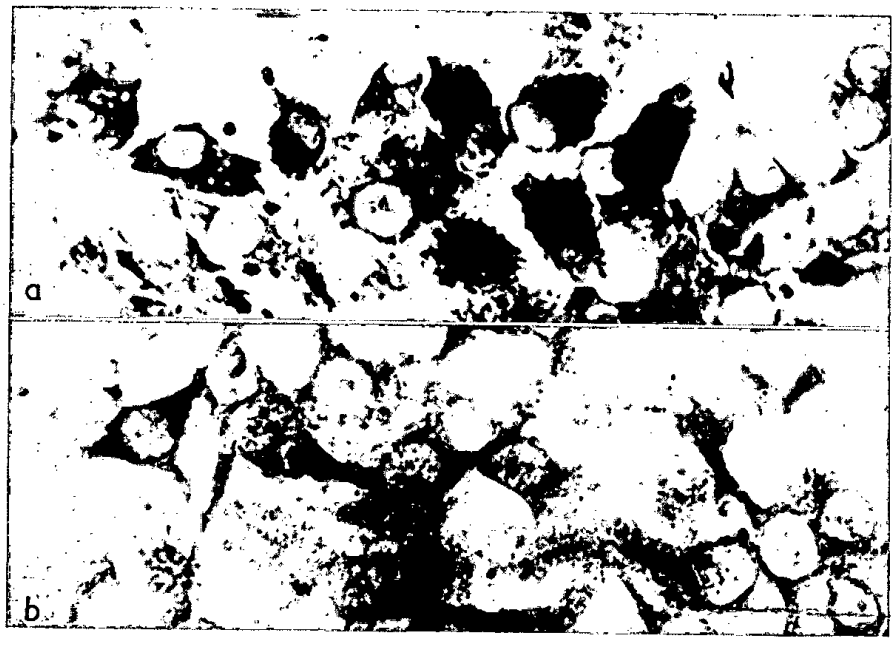

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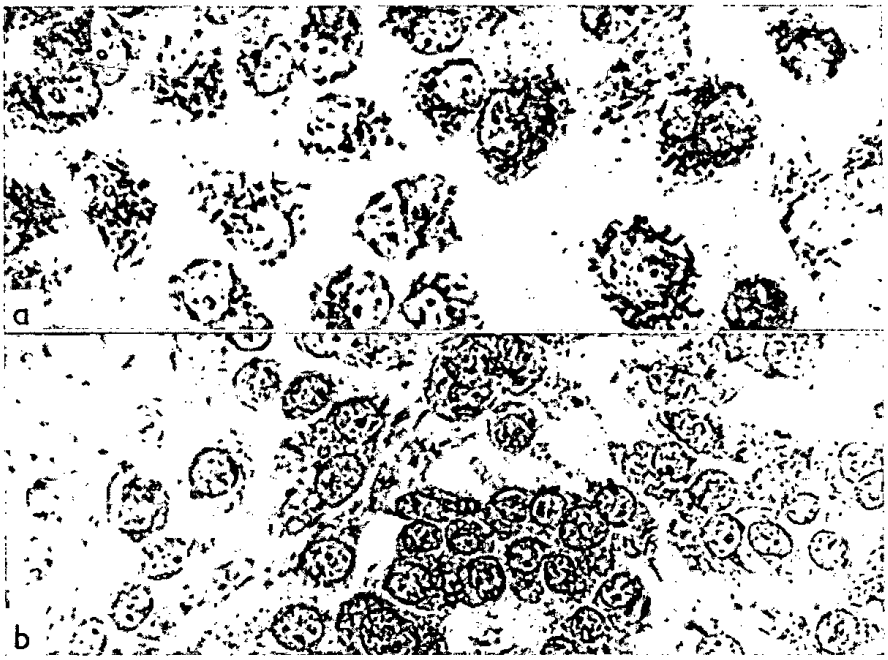

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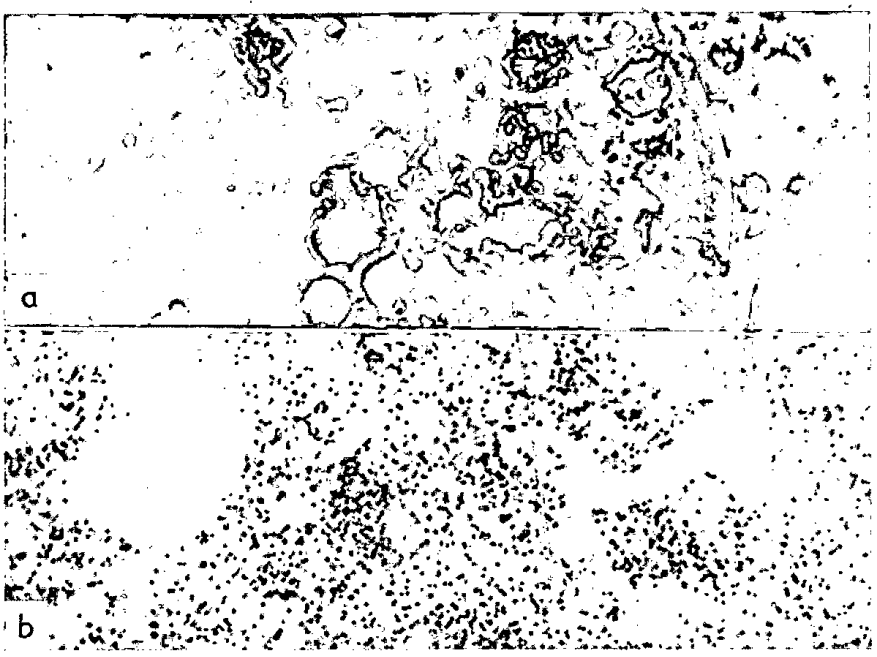

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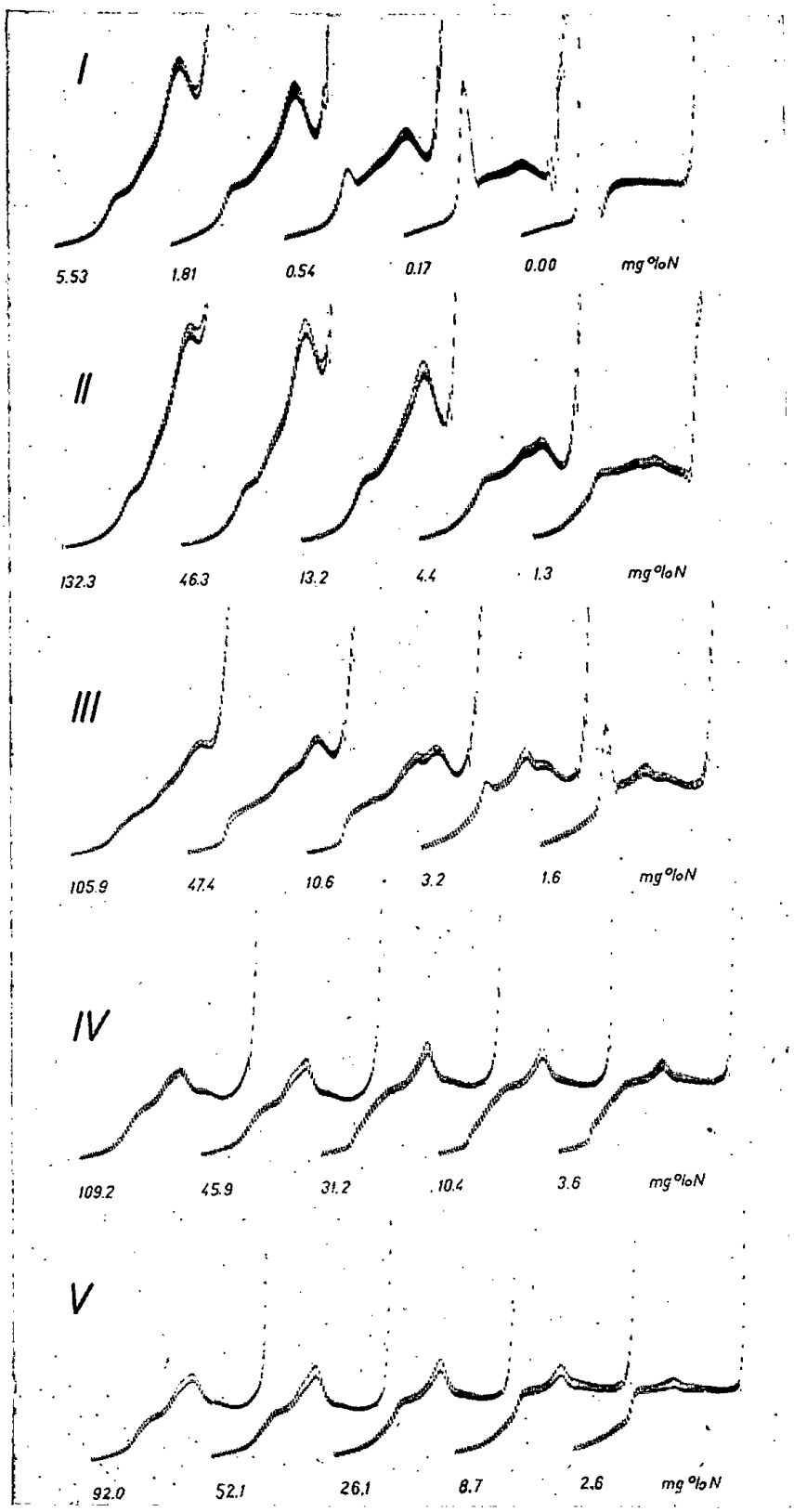

Fig. 2.
Polarographic control of purification procedure A
The samples were polarographed at 0° C in s.el. 9.5 at varying concentrations (indicated below the curves). I — IJ; II — A-TMVa_2; III — A-TMVi; IV — A-TMVg_2; V — A-TMVg_4.

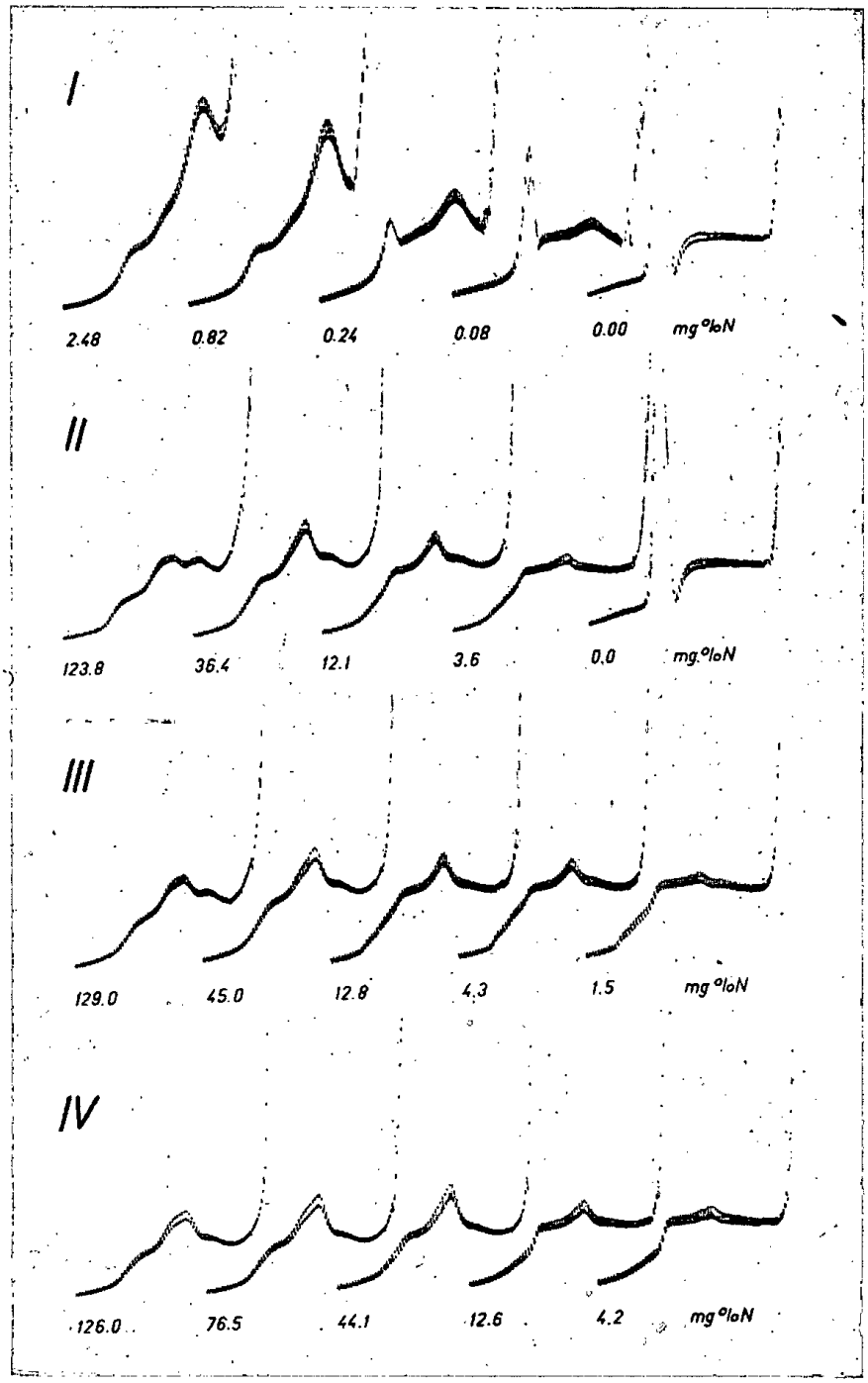

Fig. 3.
Polarographic control of purification procedure B
For explanation see Fig 2.
I — B-Sc; II — B-TMVg_2; III — B-TMVg_4; IV — B-TMVg_6.

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