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OPTIMALIZED CONDITIONS OF TICK-BORNE ENCEPHALITIS VIRUS PRODUCTION IN VITRO

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Summary. — Conditions of the efficient production of tick-borne encephalitis (TBE) virus in chick embryo cell (CEC) monolayer cultures were investigated. The prerequisites of high recovery of the produced virus appeared to be: the use of dense cell monolayers, reasonable frequency of harvests and a suitable buffering of nutrient media. Using low input multiplicity of infection, the peak of the serum-free virus production was observed between 24 and 48 hr post infection (p.i.). The procedure elaborated was useful for isotopic labelling of the virus.

Key words: *chick embryo cells*; *tick-borne encephalitis virus*; *virus production*

Introduction

CEC monolayer and/or suspension cultures were previously used for TBE virus production (Daneš and Benda, 1960; Libíková, 1969; Heinz and Kunz, 1977, 1979). However, TBE virus is a thermosensitive and pH-labile entity, which usually exhibits low cytopathogenicity in and low recovery from cultured cells.

The survival of TBE virus in nutrient media at 37 °C is not greater than a few hr (Nguyen Hong Diet, 1977); this alone represents a significant limitation of successful virus production in vitro. Further loss of the virus may arise from its inhibition by the pH values below neutral, which itself is an irreversible phenomenon (Salminen, 1962).

The purification of TBE virus produced in the suckling rat brain was previously described (Slávik, 1968; Slávik *et al*., 1970). In this paper we present details of a procedure developed for prodution of TBE virus during studies of TBE virion (Slávik and Čiampor, 1975; Blaškovič and Slávik, 1981) presuming that the capacity of CEC to produce the virus was higher than usually found.

Materials and Methods

Virus. TBE virus stock (clone P-III E) was prepared as 20% (w/v) brain suspension containing 10^8 LD_{50} per 0.4 ml and lyophilized after protamine treatment and low-speed centrifugation. One ampoule of the viral stock served for infection of 15—25 chick cell monolayers. The history and titration of the virus was described elsewhere (Slávik, 1968; Slávik *et al*., 1970).

CEC monolayer cultures. The CEC obtained from 10—11-day-old embryos were seeded in the amount of 10^8 per 150 cm^2 Petri dish in 15 ml of medium 199 or basal Eagle's medium (BEM) supplemented with 10% of calf serum. After incubation at 37 °C for 16 hr, the dense monolayers were washed once with preheated phosphate buffered saline (PBS) pH 7.2, and infected with resuspended stock virus for 90 min at ambient temperature. Then 10 ml of serum-free medium per dish was added. The virus-infected cells were incubated at 36 °C for 24 hr, thereafter dishes were cooled, medium harvested and cells were refed with a fresh nutrient. The same steps were repeated at 48 hr and the virus production was finished by the harvest at 72 hr p.i. The pooled media from three subsequent days of cultivation were treated with protamine (2.5 mg/ml-final), stored for 30 min at 4 °C and clarified for 15 min at 3,000×g.

Chemicals. The media were buffered with 10 mmol/l N-2-hydroxyethylpiperazine-N'-2--ethanesulphonic acid (HEPES, Good *et al.*, 1966) to the pH of 7.6 at 24 °C (Shipman, 1969).

Protamine sulphate (salmine, Calbiochem) 5% (w/v) stock solution was stored at —18 °C. Its pH was checked before each use and adjusted to the value between 8.0 and 8.5, when necessary. Yeast RNA, type XI was purchased from Sigma, fraction V of bovine serum albumin from Calbiochem, D_2O, bentonite and Nonidet P-40 from B.D.H.

Radioisotopic labelling. In interval between the 24th and the 48th hr p.i., the cells were supplemented with serum-free BEM containing 1/10 of respective amino acids and radioactive amino acids in the final concentration of 370 kBq/ml. The ^{14}C-glycine (specific activity = 2.6 MBq per micromole), and ^{14}C-amino acid hydrolysate (specific activity = 37 MBq per mg), were produced and supplied by Institute for Research Production and Employment of Radioisotopes, Prague.

For double-labelling of TBE virus, besides of the ^{14}C-amino acid hydrolysate, ^3H-uridine (specific activity = 65 MBq per micromole) in final concentration of 65 kBq/ml was added to the nutrient medium.

Virus production in vivo. The 1—3-day-old suckling rats were inoculated intracerebrally with resuspended viral stock (Slávik, 1968). The brains were quickly harvested after decapitation of animals and frozen on the solid CO_2 in ethanol. The suspension (10%, w/v) was prepared in 7 mmol/l Tris-HCl-buffered saline (TBS) pH 8.5; the protamine was added, and the suspension was further processed as described above for cell culture media.

Concentration and detection of the virus. The virus in the supernatants from low-speed centrifugation was pelleted in high-capacity swing-out rotors (SW-27 of Beckman L-2, or SW-23 rotor of MSE-65 centrifuges) for 90 min at 23,000 rev/min onto a cushion of 65% (w/v) sucrose in TBS. The virus banding on interphase was isolated by successive aspiration of both supernatant and cushion, diluted and stored at 4 °C prior to protamine treatment and analysis in sucrose density gradients. The concentrated virus in volume of 0.5—1.2 ml was layered over 5.0—5.5 ml preformed 10—50% (w/v) sucrose density gradient in TBS and centrifuged for 70 min at 40,000 rev/min in the SW-60 rotor of MSE-65 superspeed ultracentrifuge. After centrifugation, the total of 6.0—6.2 ml of density gradient column in lusteroid tubes of Beckman angle rotor 40 was fractionated from the bottom under OD_{254} monitoring. The peristaltic pump (type 315, Unipan, Pland), located under UV monitor (UV-Analyser, Developmental Workshops of Czechoslovak Acad. Sci., Prague), managed the through-flow of about 1 ml/minute during fractionation. The sedimentation rate of TBE virus was as previously reported (Blaškovič and Slávik, 1981), witht he only difference that the viral peak there corresponded to the virus pelleted at the bottom of the tube instead of the cushion.

The viral mass in the peaks was calculated according to the calibration curve made with the mixture of 7% RNA and 93% of bovine serum albumin, and applied in different concentrations to the UV monitor. A good correlation with the direct protein estimation (Lowry *et al.*, 1951) in the peak fractions was observed.

Electron microscopy. A drop of the sucrose peak fraction was dialyzed overnight against 2 mmol/l Tris-HCl buffer pH 8.5 at 4 °C. The sample was applied onto a carbon coated perforated formvar grid and stained for 1 min with 1% (w/v) phosphotungstic acid, pH 7.3. The pictures were taken on Phillips EM 300 at 70,000-fold instrumental magnification.

Isolation of envelope and capsid fractions of the virus. The double labelled virus isolated from sucrose density gradient was diluted 1 : 1 with TBS, and it was layered over 3 ml of sucrose in D_2O containing 0.85% sodium chloride, 7 mmol/l Tris-HCl pH 7.8, and 1 mg/ml bentonite. The sucrose-D_2O part of the column consisted of 1 ml of 75% (w/v) sucrose, 1 ml of 38% sucrose, and 1 ml of 35% sucrose containing 0.25% Nonidet P-40. The centrifugations were carried out for 1 hr at 40,000 rev/min and for 2 hr at 59,000 rev/min, respectively, in the rotor SW-60 of

MSE-65 centrifuge. The construction of density gradient column and the detergent treatment of the virus during centrifugation were found effective for the quick and high-recovery isolation of TBE virus capsid fraction. The densities of fractions were estimated refractometrically according to the calibration curve constructed with weighed sucrose-D_2O solutions. Prior to the counting in the Packard TriCarb, model 3330 scintillation spectrometer, the gradient fractions were processed as previously described (Slávik *et al.*, 1976). The samples were counted at the efficiencies of 39%, and 67% of the ^3H-, and ^{14}C-standard, respectively. The spill-over of the carbon to the tritium channel was subtracted. The pooled fractions of the peaks from the density of 1.32 $g.cm^{-3}$ and of about 1.20 $g.cm^{-3}$ of sucrose-D_2O were dialyzed overnight at 4 °C against TBS, pH 7.5. The former sample was concentrated by centrifugation for 2 hr at 36,000 rev/min in SW-60 rotor, the latter on PM-30 membrane of Amicon, model 12 stirred cell. The samples were separately analysed in polyacrylamide gels.

Polyacrylamide gel electrophoresis (*PAGE*). The structural polypeptides of TBE virus were analysed in 8% gels as previously described (Slávik *et al.*, 1976). The modified Schiff's staining procedure was that of Zacharius *et al.* (1969).

Results and Discussion

Conditions selected for the production of TBE virus in vitro

The TBE virus was produced in CEC monolayer cultures at the input of 0.03 – 0.05 LD_{50} per cell in order to work with a lyophilized precisely titrated virus. The optimal pH for cultivation of CEC has been reported between 6.95 and 7.3, with some cellular growth at 7.6 (Ham and McKeehan, 1979). The chosen pH of HEPES-buffered media, i.e. 7.6 at 24 °C, may shift to the pH of 7.32 at 36 °C, and to 7.77 at 4 °C (ΔpK_a/°C of HEPES =

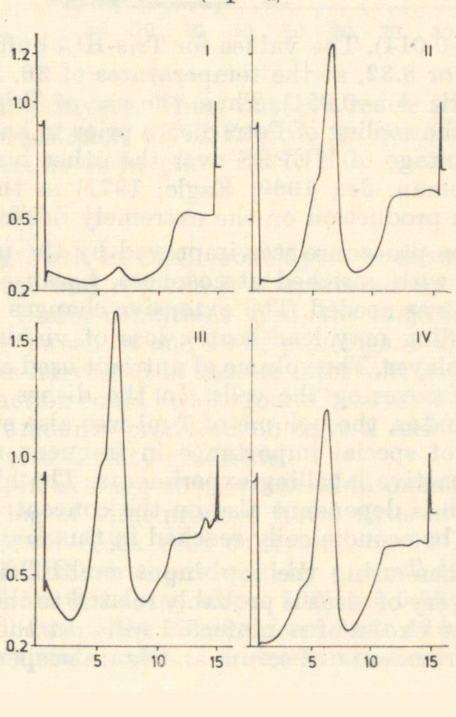

Fig. 1.

Analysis in sucrose density gradients of TBE virus amounts produced by chick cells in three subsequent days p.i.
I — virus detected in medium harvested at 24 hr p.i.;
II — one third or the virus harvested in the interval between 24 and 48 hr p.i.;
III — two thirds of the virus material narvested as above;
IV — virus detected in the medium harvested at 72 hr p.i.
Abscissa: fraction number; ordinate: O.D. at 254 nm.

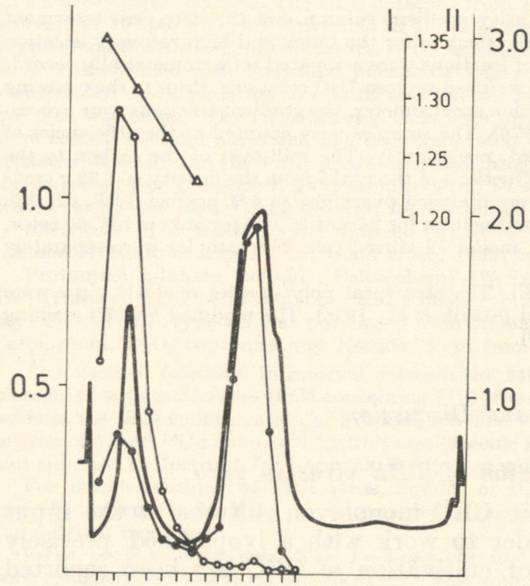

Fig. 3.

Effect of Nonidet P-40 treatment on double labelled TBE virus in the sucrose-D_2O density gradient column ^{3}H-radioactivity ○——○, ^{14}C-radio-activity ●——●, OD_{254} ——, density ($g.cm^{-3}$) △——△.
Abscissa: fraction number; left ordinate: OD_{254}; right ordinates: I — density; II — counts/min × 10^{-4}.

= −0.014). The values for Tris-HCl buffered media ranged from 7.32 up to 7.69 or 8.32, at the temperatures of 36, 24, and 4 °C, respectively (ΔpK_a/°C of Tris = −0.031). Thus, the use of Tris-buffered media is indeed possible, but the cooling of Petri dishes prior to harvesting is not desirable. The major advantage of HEPES over the other buffers is its higher buffering capacity (Shipman, Jr., 1969; Eagle, 1971) in the pH range suitable for the TBE virus production on the extremely dense CEC monolayers.

The procedure was improved by the use of ordinary incubator instead of that with enriched atmosphere, because the frequent handling of cultured cells was needed. The extensive changes of the overlay pH in the course of refeeding may lead to the loss of viable cells and to local acidification in monolayer. The volume of nutrient used and applied per dish formed a 0.7 mm layer covering the cells. In the dishes held by well equilibrated trays of incubator, the volume of 7 ml was also sufficient. The small overlay volume was of special importance in frequent nutrient changes, and also for the radioactive labelling experiments. The high specific activity of the product, which is dependent also on the concentration of the radionuclid in medium may be economically reached in this manner.

When using the low input multiplicity of infection, the low first day recovery of virus is probably related to the readsorption of the virus produced to the excess of noninfected cells. At the same time, the non-attached cells and remnants of serum and brain suspension from viral stock, are removed

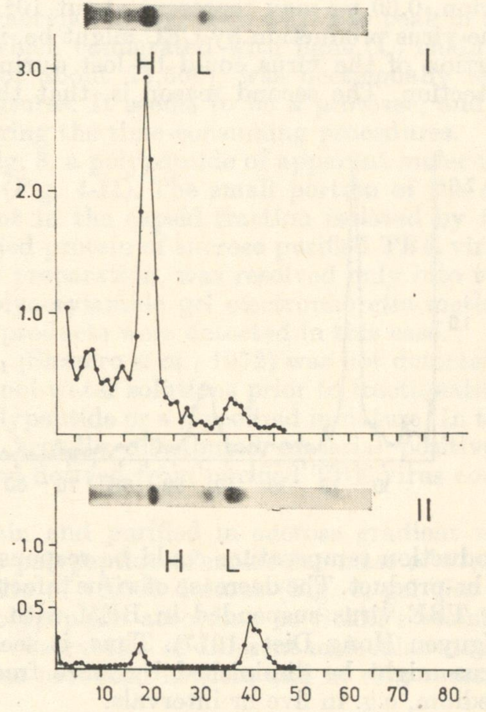

Fig. 4.
PAGE of Nonidet P-40 resolved TBE virus fractions
I — Material concentrated from fractions No. 10—12 on Fig. 3.
II — Material pelleted from fractions No. 2—4 on Fig. 3. The inserts are gels stained and photographed prior to fractionation and counting. In addition to the viral polypeptides, the gel also contains heavy (H) and light (L) chains of rabbit immunoglobulin.
Length of inserts = 0.7 × length of the graphs.
Abscissa: fraction number; ordinate: counts/min × 10^{-3}

from the virus-producing cellular monolayers. Thus, one can reach a good approximation to the serum-free production of TBE virus in vitro, when changing the nutrient at 24 and then at 48 hr p.i.

The production of TBE virus by CEC

A typical picture of TBE virus growth in CEC monolayers is shown on Fig. 1. The amount of virus produced and recovered is expressed by viral peaks in sucrose density gradients. Under conditions used, the majority of virus was produced in the interval between 24 and 48 hr p.i. (Figs. 1-II and 1-III). However, when the virus was harvested in shorter intervals, in some experiments, the peak of virus production could be also found between 44 and 66 hr p.i. The proportion of virus amounts produced on days 2 and 3 p.i. was 70% to 26% as detected in sucrose density gradients.

From the 10^8 control cells seeded, in average 85% remained in monolayer after 48 hr of cultivation. The 1.1 mg of viral product (total virus in Figs 1-I-IV) originated from about 1.3×10^9 chick cells cultivated on 15 Petri dishes. It corresponded to 0.86 pg of TBE virus produced by one cell during the whole 72 hr cultivation period. From this value, about 70%, i.e. 0.60 pg of virus, was produced by one chick cell during the interval from 24 to 48 hr p.i. When accepting 50×10^6 as an approximate value of molecular mass of TBE virion, 0.60 pg may represent about 10^4 particles originating from one cell. The virus production by CEC might be, in fact, significantly higher, as some portion of the virus could be lost during its purification, and thus escaped detection. The second reason is, that thermal inactivation of virus at the

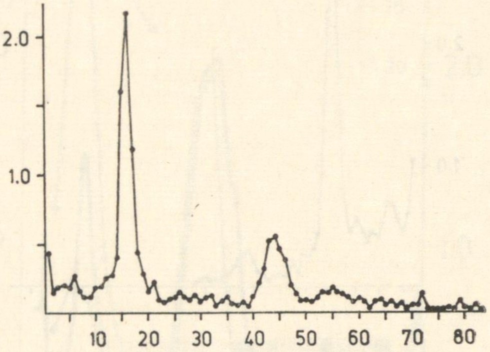

Fig. 5.

PAGE of ^{14}C-glycine labelled TBE virus Medium from infected CEC was harvested at 48 hr p.i. and purified in the sucrose density gradient.
Abscissa: fraction number; ordinate: counts/min $\times 10^{-3}$.

production temperature could be responsible for a loss of about 50% of the 24 hr-product. The decrease of virus infectivity by two $\log_{10}$ has been reported for TBE virus suspended in BEM with 10% serum during 17 hr at 37 °C (Nguyen Hong Diet, 1977). Thus, it seems that thermoinactivation of the virus might be diminished by more frequent harvests of virus containing medium, e.g. in five hr intervals.

The perfusion system is perhaps too complicated and hazardeous for laboratory scale virus production.

Electron microscopy

In the viral preparations purified from CEC, the spherical particles with external diameter of 45—60 nm in average were observed (Fig. 2). The particles with greater external diameters appeared to have a disrupted envelope, and in that case, the stain penetrated into the particle interior. These envelope structures were seen as lighter zones marginated with the morphological envelope subunits.

Characterization of the viral product by radiochemical techniques

The TBE virus double labelled with ^3H-uridine and ^{14}C-amino acids yielded two fractions upon treatment with Nonidet P-40. Density of the RNA-rich fraction was about 1.32 g. cm^{-3}, while most of the ^{14}C-radioactivity banded in the low density part of the sucrose-D_2O density gradient column (about 1.20 $g.cm^{-3}$, Fig. 3). The broadening of the radioactivity peaks and their shifts to higher densities, when compared with the records of optical density shown on Fig. 3, were due to the effect of peristaltic pulsations on the fractionation. The pooled fraction No. 10—12 contained material comigrating with the heavy (H) chain of immunoglobulin (molecular mass of about

50,000) upon electrophoresis in polyacrylamide gel (Fig. 4-I). The peak in the fraction No. 34–37 on Fig. 4-I, which comigrated with light (L) chain of immunoglobulin (molecular mass of about 23,500), was occasionally seen in viral preparations form CEC cultures. It seems to be a protease, and/or self break-down product arising during the time-consuming procedures.

In the fractions No. 2–4 from Fig. 3, a polypeptide of apparent molecular mass of 16,000 daltons was found (Fig. 4-II). The small portion of the envelope polypeptide was also present in the capsid fraction isolated by the method used. The ^{14}C-glycine labelled protein of sucrose purified TBE virus, which was analysed instantly after preparation, was resolved only into two main viral polypeptides by the polyacrylamide gel electrophoretic method employed (Fig. 5). No break-down products were detected in this case.

The material corresponding to V_1 (Shapiro *et al.*, 1972) was not detectable in gels stained in acetic acid-methanol-water solutions prior to fractionation. It could be either a hydrophobic polypeptide or a glycolipid in nature. In this region of the polyacrylamide gel, broadly distributed material positively stained with the Schiff's reagent and derived from purified TBE virus could be observed (results not shown).

The TBE virus produced in brain and purified in sucrose gradient was resolved in PAGE into an envelope polypeptide of molecular mass of 57,000 daltons, and to a capsid polypeptide of 16,000 daltons (Fig. 6, gel No. 1). The former was a glycosylated polypeptide according to Schiff's staining (Fig. 6, gel No. 2). The reason of the observation of different molecular masses for TBE virus envelope polypeptides produced in vitro and in vivo remains obscure, and needs further study.

The TBE virus produced in CEC monolayer cultures was shown herein to be a particulate RNA containing material with defined structural polypeptides. Why the virus growth declines on the 3rd day p.i. is not known. Besides the nutrient starvation of the cells and possible effect of interferon (Stanček, 1965; Libíková and Rajčáni, 1974), also some other factors may play a role.

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References

Blaškovič, D., and Slávik, I. (1981): Fine structure of tick-borne encephalitis virus, pp. 133—141. In Ch. Kunz (Ed.): *Tick-borne encephalitis.* Facultas, Vienna.

Daneš, L., and Benda, R. (1960): 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—36.

Eagle, H. (1971): Buffer combination for mammalian cell cultures. *Science* **174**, 500—503.

Good, N. E., Winget, G. D., Winter, W., Conolly, T. N., Izawa, S., and Singh, R. M. M. (1966): Hydrogen ion buffers for biological research. *Biochemistry* **5**, 467—477.

Ham, R. G., and McKeehan, W. L. (1979): Media and growth requirements. *Meth. Enzymol.* **58**, 44—93.

Heinz, F., and Kunz, Ch. (1977): Concentration and purification of tick-borne encephalitis virus grown in suspensions of chick embryo cells. *Acta virol.* **21**, 301—307.

Heinz, F., and Kunz, Ch. (1979): Dissociation of tick-borne encephalitis virus by Triton X-100 and cetyltrimethylammonium bromide. *Acta virol.* **23**, 189—197.
Libíková, H. (1969): Interaktion des ZE-Virus mit der Zelle bei Züchtung in vitro, pp. 81—90. In H. Libíková (Ed.): *Virus der Zeckenencephalitis. Biologische Kentnisse und ihre praktische Anwendung.* Publishing House of Slovak Academy of Sciences, Bratislava.
Libíková, H., and Rajčáni, J. (1974): Experimental double infection with tick-borne encephalitis and herpes simplex (type 1) viruses. *Acta virol.* **16**, 47—56.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951): Protein measurement with Folin phenol reagent. *J. biol. Chem.* **193**, 165—175.
Nguyen Hong Diet (1977): Experimental mixed infection with Toga-, Arena- and Herpetoviruses, p. 86. Dissertation Thesis, Institute of Virology, Slovak Academy of Sciences, Bratislava.
Salminen, A. (1962): Chemistry of nonspecific innibitors of hemagglutination by arthropod--borne viruses. *Virology* **16**, 201—203.
Shapiro, D., Trent, D., Brandt, W. E., and Russel, P. K. (1972): Comparison of the virion polypeptides of group B arboviruses. *Infect. Immun.* **6**, 206—209.
Shipman, Ch., Jr. (1969): Evaluation of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as a tissue culture buffer. *Proc. Soc. exp. Biol. Med.* **130**, 305—310.
Slávik, I. (1968): Studies on tick-borne encephalitis virus. I. Partial purification of virus from rat brain suspensions. *Acta virol.* **12**, 535—545.
Slávik, I., Mrena, E., and Mayer, V. (1970): Studies on tick-borne encephalitis virus. II. Virus morphology and some data on virus structure. *Acta virol.*, **14**, 8—16.
Slávik, I., and Čiampor, F. (1975): Proteins of tick-borne encephalitis virus, p. 270. In H. S. Bedson, R., Najera, L. Valenciano and P. Wildy (Eds): *International Virology 3.* Abstracts of 3rd Internat. Congr. for Virology.
Slávik, I., Krasnobayeva, Z. N., Gaidamovich, S. Ya., and Melnikova, E. E. (1976): Composition of the Zaysan virion. *Acta virol.* **20**, 177—182.
Stanček, D. (1965): The role of interferon in tick-borne encephalitis virus-infected L cells. III. The effects of temperature on the production of virus and interferon by L cells during acute and persistent infection. *Acta virol.* **9**, 298—307.
Zacharius, R. M., Zell, T. E., Morrison, J. H., and Woodlock, J. J. (1969): Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **30**, 148—152.

Explanation of Figures (Plates IX—X):

Fig. 2. Negative staining of the TBE virus produced in vitro, magnification × 280,000.
Fig. 6. PAGE of the TBE virus proteins produced in the brains of suckling rats.
1 = the gel containing 60 μg of protein stained with Coomasie brilliant blue R-250.
2 = 200 μg of viral protein electrophoresed and stained with modified Schiff's procedure.
3 = Monomer and oligomers of the horse heart cytochrome C stained with Coomasie brilliant blue R-250 (molecular mass of the monomer about 12,800).
4 = Blank of solvents.