

Registration of spontaneous photon emission from virus-infected cell cultures: Development of experimental system

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"Viruses are probes by which one can gain insight into cellular structure and function".

Sir M.F. Burnet

Detection of spontaneous photon emission from virus-infected cells was attempted using cell monolayer cultures prepared from the established cell lines differing by origin and sensitivity to viruses. The experimental system was elaborated permitting maintenance of the cell monolayer cultures grown upon quartz slides placed inside quartz cuvettes within the photomultiplier chamber during prolonged time periods (till 24-36 hr) covering the whole virus multiplication cycle. Rich nutritive medium was employed, providing undisturbed cell viability and virus-induced cytopathic effect (CPE) development during such prolonged experiment, each ingredient of the medium being checked as potential parasitic emitter or extinguisher of the cell-specific emission. As presupposed 'positive control', the *in vivo* cultivated chorio-allantoic membranes (CAM) of 10-days-old chick-embryonated eggs were used. The virus-infected CAMs showed specific peculiarities of the emission dynamics as compared to monotonous dynamics shown by non-infected CAMs. Similar dynamic regularities were observed in cell monolayer cultures containing much lesser (by order) number of cells per exposed sample. Using the elaborated system, some specific changes in the virus-infected cells were found, being correlated with two stages of virus replication cycle: the initial stage, synchronous penetration of the pre-adsorbed virus inside the cell, and a later stage, characterized by intensive CPE manifestations.

Keywords: Biophotonics, Cell monolayer culture, Holistic principle, Mitogenic radiation, Photon emission, Viral inoculation, Virus-infected cells.

The modern virology plays a vanguard role in the whole field of molecular genetics—the leading discipline of molecular and cell biology—which is the dominating trend in the life sciences. Apart from highly important practical aspects connected with the viruses as infectious agents causing highly dangerous diseases in humans ranging from influenza devastating pandemics to the incurable fatal AIDS, the virus research is of great theoretical importance. This is due to unique advantages of the viruses as experimental tools. The fundamental fact is that the viruses are obligate parasites being able to grow and multiply only within living cells and, thus, becoming involved into the very basic intracellular machinery determining cellular structure and function. The virological approach in molecular biology covers all the kingdoms of living species including both the prokaryotic ones, e.g. bacteria, and the eukaryotic ones, e.g. protists, fungi, plants, and animals. The

progress in virus research has become closely interrelated with the advancement of biotechnology, providing highly sophisticated equipment ensuring high level of experimental studies. As a result, the molecular biology has demonstrated an extraordinary rate of accumulation of scientific facts together with their rapid practical application. However, there is a paradoxical situation when the great progress in experimental studies is combined with poor and rigid theoretical basis. Essentially, the main theoretical paradigm is based on the triplet code dogma, which, having been formulated almost half a century ago, has not been developed further and still is considered as the highest achievement of the biological thought. Accordingly, the extensive experimental studies were not followed by intensive theoretical development.

The present studies are based on the employment of the viruses in especial field of biophysics, namely, biophotonics, which is a modern term^{1,3} for the mitogenetic doctrine based on the phenomenon of mitogenetic radiation discovered by A. Gurwitsch^{4,5} in 1923. This field which seems to be 'on the kerb' of

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the main stream of the nowadays biology, stands on the theoretical grounds^{4-8,9-12} quite different from the dominating genetic basis. The most important theoretical ground of the biophotonics in the view of the present context is based on holistic principle¹³⁻¹⁷. In accordance with it, the advantages of the proposed research based on application of viruses in the field of biophotonics are to be considered.

Advantages of using viruses in biophotonics field:

The whole chain of events starting from the first contact of an infectious viral particle (virion) and ending by the release of newborn virions from the infected cells, which is designated as virus multiplication cycle is characterized by the disjunctive way of reproduction. This means that different viral components are synthesized separately (spatially and temporally) within the infected cell. Thus, the cycle includes the following main stages:

- 1 attachment of the virions upon the cell surface;
- 2 penetration of the attached virions inside the cell;
- 3 uncoating—intracellular release of the viral genetic material;
- 4 transcription and translation of the input virus genome;
- 5 switch off (at least partly) the host cell genome-coded synthesis and switch on the viral genome-coded intracellular synthesis;
- 6 synthesis and accumulation of the virus-specified nucleic acid;
- 7 translation of the newly synthesized viral nucleic acid, i.e. synthesis of the viral polypeptides;
- 8 post-translational modifications of the viral polypeptides—functional “maturation” of the viral proteins;
- 9 virus assembly: Transport of the viral components via intracellular “traffic” and formation of the virions;
- 10 induction of the cytopathic effect (CPE): Necrotic and apoptotic modes of the virus-induced cell death; and
- 11 release of the newly formed virions outside the cell.

The above list clearly displays the wide scope of unique advantages of the viral models for their employment in the biophotonics field.

Advantages of application of the biophotonics field for virus research:

The above chain of events constituting the virus multiplication cycle is usually analyzed using either

chemical or genetic parameters, the latter being reduced to the former. This means that the triplet genetic code is not accounted as ontological essence while the studies are concentrated on the mechanism of the code realization, i.e. the sequence of enzymatic chemical reactions involved into transcription/translation processes determining specificity of the synthesized proteins. Such reductionistic analysis of the virus multiplication cycle is a traditional way of accumulation of knowledge concerning cellular structure and function. However, the ontological essence of the discovered triplet code would mean that the nucleotide combinatorics within a codon (three nucleotide bases coding for an amino acid) as well as the codons' combinatorics within a gene cannot be in principle inferred from any physical-chemical properties of the nucleotides themselves.

The suggested “virus-biophotonics marriage” to be used as an experimental approach for understanding living expressions means a combination of the holistic principle with analytic parcellation based on the reductionist mode. A specific purpose of this approach consists in attempt of dynamic registration of spontaneous photon emission from virus-infected cells and confrontation of the experimental results obtained on biophotonic level with the enormously extensive source of knowledge obtained on biochemical and genetic levels. Such confrontation would permit to connect different dynamic characteristics of the photon emission (e.g. intensity and, especially, rhythmicity) with different stages of the above-described virus multiplication cycle that have been studied in detail. The cell cultures predominantly employed in virology as experimental “hosts” for viral infection, apart from the empirical convenience, have some advantages which are unique for the considered experiments. The main point is that the cell culture is a cloned population of homogeneous cells, contrary to a multicellular organism in which the cells are structurally and functionally differentiated. Therefore, the photon emission to be registered from the cell culture may be considered as a result of a total effect related to all the practically equivalent members (cells) of the population.

Materials and Methods

Virus—Two viruses were used (1). The strain Australia-Victoria of the Newcastle disease virus (NDV) taxonomically belonging to the family Paramyxoviridae, order Mononegavirales, is RNA-

containing virus with non-segmented genome of negative sense (i.e. the virion RNA is complementary to the virus-specified intracellular messenger RNA) encoding for three envelope glycoproteins and three 'core' proteins^{18,19}; (2). The Rostock strain of fowl plague virus (FPV) which is avian influenza virus taxonomically belonging to the family Orthomyxoviridae²⁰ is RNA-containing virus with segmented genome of negative sense encoding for three envelope proteins and five 'core' proteins. The virus was propagated in 10-days-old chick embryonated eggs, the virus-containing allantoic fluid was harvested 72 hr after inoculation of the eggs into allantoic cavity. The titration of the virus was performed by hemagglutination (HA) reaction using 0.5% of chick red blood cells. The virus used in the main experiments was purified and concentrated by differential centrifugation including: (1) clarification of the allantoic virus (sedimentation of the cell debris) by centrifugation at 5000 g; (2) sedimentation of the virus in ultracentrifuge at 40000 g (pellet of the "crude" virus); and (3) 30-60% sucrose gradient centrifugation of the crude virus at 50000 g (highly purified virus). The virus-containing band was collected, homogenized in PBS, sonicated, diluted up to hemagglutination titer of 1:5000 with PBS, pre-packed in 1 ml quantities and stored at 20°C until used. In each experiment, a new sample of the pre-packed virus was used.

Cells—Two cell systems were used in the studies: (1) *in vitro* cultivated chorio-allantoic membranes (CAM) prepared from chick-embryonated eggs, and (2) the established lines of the cell cultures of different origin.

The CAMs were prepared *ex tempore* just before the experiment from 11-days-old chick-embryonated eggs²¹. They were cut off from the embryo body, separated from the internal surface of the egg shells, washed thrice in phosphate-buffered saline (PBS), and put into Petri dishes (10 cm diam.) with 20 ml PBS.

The cell cultures used were BHK, 'Baby Hamster Kidney', epithelial origin; VERO, monkey kidneys, epithelial origin; CV-I, human conjunctiva, epithelial origin; and H9C2, heart myoblast, mesoderm origin.

The BHK, VERO, and CV-I cell lines were obtained from the Institute of Virology, Marburg University, Germany (Prof. H-D. Klenk), H9C2 cell line was obtained from the Department of Molecular Cell Biology, Utrecht University, The Netherlands (Prof. R. van Wijk).

The cultured cells were grown as monolayer cultures in plastic flasks (Falcon Co.) using the L15

nutrition medium (Difco Co.) with addition of the 10% calf serum. After formation of confluent monolayer, the cells were washed twice with PBS and treated with the trypsin-versene (EDTA) mixture (0.01/0.002%). After the cell detachment, the medium with the detached cell culture sheets was shaken vigorously in order to break the sheets and obtain an appropriate cell suspension consisting of separate cells. The concentration was adjusted by PBS to 100,000 cells/ml that was used for further cell propagation. The conditions of the cell maintenance were elaborated during the experiments and described in the section 'Results'. For the experiments themselves, the cell monolayer cultures were grown upon quartz slides placed into plastic Petri dishes (5 cm diam.). The composition of the nutrition medium was an objective of the preliminary part of the studies, the purpose being to enrich the medium as much as possible with nutrient components that will be as neutral as possible as potential luminescence sources which might interfere with the intrinsic cell-induced photon emission.

Viral inoculation—The viruses were appropriately diluted in PBS to provide multiplicity of infection of 20 (i.e. a number of infectious viral particles per cell to be infected) securing synchronous infection of all the cells.

Before the virus inoculation, both the *ex tempore* prepared CAMs and the monolayer cultures grown upon quartz slides within Petri dishes were washed twice with PBS.

The two CAMs were put into the quartz cuvette and inoculated by the virus using 15 ml viral preparation to provide the above-described multiplicity of infection (20 virions per CAM's mesothelial cell) and the cuvette was placed into conditions of 4↔ C for 1 hr. After that, the viral inoculate was sucked out, the infected CAMs were washed twice with precooled PBS (to get rid off the excess of the unadsorbed virus), a new portion of the pre-heated maintenance medium (20 ml) was added, and the cuvette was placed into the photomultiplier chamber (37°C). After the adsorption period, the viral inoculate was sucked out, the slides with the infected cell monolayers were washed twice with the PBS (to get rid off the excess of the unadsorbed virus), the slides were put into the quartz cuvette with 20 ml of the pre-heated maintenance medium, and the cuvette was placed into the photomultiplier chamber 37°C for further registration of the photon emission.

Photon registration—The photon registration was carried out by two photomultipliers: either PMS-1

with one chamber, or PMS2 with two adjacent chambers, the latter permitting simultaneous registration from two different samples, e.g. non-infected and virus-infected cells, etc. The cathodes' sensitivity was within the range of 200-800 nm. All the technical details of the photomultipliers are described in detail elsewhere²². The cells grown upon the quartz slides were placed into quartz cuvette filled with an appropriate maintenance nutritive medium. The elaboration of conditions permitting cell maintenance inside the chamber during the whole multiplication cycle was one of the main objectives of the preliminary part of the research and described under results. The registration of the current photon emission was performed by adjustment to the Statistica computer program.

Results

The whole studies included preliminary and main parts.

The preliminary part pursued two aims. The first one was to elaborate optimal conditions providing detection of the photon emission unbiased by any ingredients to be included into the cell culture maintenance medium. Such ingredient(s) may turn out to be either a source or inducers of any 'parasitic' luminescence, on one hand, or 'extinguishers', i.e. either absorbing the intrinsic cell-originated photon emission, or inhibiting its source inside the cells, on the other. The second aim of the preliminary part was to elaborate conditions permitting an optimal maintenance of both the non-infected and virus-infected cells inside the PMS chamber for an appropriately long period, that providing continuous dynamic registration of the photon emission during the whole virus multiplication cycle which lasts up to 22-36 hrs depending on virus-cell culture combination.

I. Elaboration of experimental system

1. *Elaboration of the cell cultivation conditions appropriate for the photon measurement*—The most important advantage of the cell monolayer cultures as compared to the cell suspension was the possibility to observe microscopically the cells' viability, and the development of the CPE in the virus-infected cells and the possibility for varying spatial orientations of slides with the cell monolayers for optical arrangements and manipulations. It could be suspected that the emission intensity in the cell monolayer culture may be negligible to be detected

due to incomparably lesser (by order) amount of cells per sample as compared to any organ, or tissue, or cell culture suspension.

However, no significant difference between the emission intensities in suspension and monolayer cultures was found when the PBS as the simplest maintenance medium was used in the preliminary experiments (see the next paragraph). Therefore, since the potential advantage of the suspension culture—the possibility for the manipulations with the cell amount—was not clearly expressed, the further experimental strategy was oriented to the usage of the monolayer cultures.

2. *Elaboration of the optimal nutritive medium*—The cells were grown using the optimal L15 medium added with 10% calf serum. The strategy for the elaboration of the optimal maintenance medium was based on its stepwise enrichment with different ingredients. The aim was to select the richest medium, which would be optimal for the established conditions of the cell cultures' maintenance without significant nonspecific influence on the intensity of the registered photon emission.

As a starting point for this series of the experiments, the PBS was tested as the simplest cell maintenance medium.

It was observed that PBS medium, the cell viability was limited to 8-10 hr depending on the kind of the culture. Therefore, the measured emission was considered as a background for the next steps of the medium enrichment, which were as follows:

- I Gradual nutritive enrichment of the maintenance medium
 1. Hanks balanced salt solution;
 2. Medium containing all the amino acids (L15 medium);
 3. L15 medium plus 2% calf serum.
- II The addition of the neutral red—a vital dye and a pH indicator staining only alive cells—providing the possibility to observe cell vitality in the course of the virus-induced cytopathic effect.
- III Addition of the HEPES buffer for pH stability during long cell maintenance.
- IV Providing the sterility condition: addition of antibiotics (penicillin, streptomycin, gentamycin).

Each of the above components would have been suspected as a potential factor influencing the photon emission. In this respect, the neutral red as a colorful substance was of especial suspicion. However, neither

the media themselves, nor in combination with the cell monolayer cultures did show any significant difference in the photon emission during a 1-hour-long measurement as compared to simplest PBS medium.

Therefore, the well-established medium recommended for the optimal maintenance of the virus-infected cell monolayer cultures (Biological Industries Ltd., 2002) containing the L15 nutritive medium supplemented with the 2% calf fetal serum, neutral red, HEPES and gentamycin as antibiotic was used in the main experiments.

Procedure of the inoculation of the cells with the virus—After a series of preliminary experiments, the following procedure of the inoculation was employed:

- 1 Growth of the cell in plastic flasks (Falcon Co., diam. 5 cm) with the quartz glass slide placed at the bottom of the dish until a confluent cell monolayer is formed especially upon the surface of the slide;
- 2 washing of the cells twice by the pre-cooled PBS;
- 3 inoculation of the cells with 0.5 ml pre-cooled virus of the HA titer of 1:100 using PBS as a diluent; inoculum virus must be prepared each time from the stock virus stored at -20°C ;
- 4 incubation of the infected cells at 4°C for 1 hr;
- 5 discarding of the inoculum and washing of the cell monolayer with the pre-cooled PBS;
- 6 addition of 2.5 ml of the pre-warmed maintenance medium and incubation of the infected cells at 37°C ;
- 7 the non-infected cells underwent the same procedure, the PBS instead of the virus being used.

4. CPE expression and virus accumulation in the infected cells—In the first series of the experiments, the above-listed cell cultures were inoculated with the virus, the inoculated cells were observed microscopically, and the cytopathic effect was registered (Fig. 1). The virus accumulation in the infected cell cultures was measured at the end of the experiments, being estimated by the virus hemagglutinin (HA) titer²³. For this aim, the nutritive maintenance medium was collected, the cells were washed with the PBS buffer, scrapped from the flask surface with the rubber policeman, and homogenized by means of the Potter-Elvehjem homogenizer with teflon pestle. Both the medium and the homogenate were taken for determining the HA titer of extracellular (medium) and intracellular (cell homogenate) virus²⁴.

The results have shown strongly pronounced CPE in BHK, VERO, and CV-1 cell lines, although there were some cell line-specific differences concerning the dynamics of the CPE development and its cytological expression. In H9C2 cell line, the CPE was weakly expressed with late emergence and slow dynamics, so that the CPE was incomplete even at 36 hr p.i. (Table 1). The virus accumulation was somewhat compatible with the respective indications of the virus accumulation expressed in the hemagglutinin titer: there was high yield of the virus even in the nutritive medium (extracellular virus) in all the CPE-expressing inducing BHK, VERO, and CV-1 cell lines, while very poor accumulation in H9C2 cell line (Table 1).

In the Fig. 2, it is possible to see the development of the cytopathic effect in the case of the VERO-infected cells.

5. Elaboration of conditions of maintenance of infected cells in course of the measurements of photon emission—The purpose of these experiments was to elaborate conditions for the maintenance of the virus-infected cells within the photomultiplier chamber, so that the measurement of the photon emission could be carried out *in situ* immediately after infection and then continuously during the whole virus multiplication cycle. It was desirable not to open the chamber during the experiment performance lasting 24-36 hr. The conditions included the stable maintenance of the temperature (37°C), sufficient ventilation, and humidity—all that had to be combined with the absolute darkness conditions. These conditions have been achieved by the necessary technical adjustments. As a result, the comparative investigation of the non-infected and virus-infected cells maintained in the thermostat conditions outside the photomultiplier, on one hand, and those maintained within the photomultiplier chamber, on the other, was carried out. The criteria of the estimation included the cell vitality, the development of the virus-induced cytopathic effect, and the HA titer of the newly formed intracellular virus.

Table 1—CPE development and virus yield in the infected cell cultures

CPE development (Time pi in hr)	Virus yield expressed in hemagglutinin titer	
	Intracellular	Extracellular
1. BHK 12-24, complete	1:32	1:4
2. VERO 16-26, complete	1:64	1:2
3. CV-1 18-28, complete	1:32	1:4
4. H9C2 24-36, partial	1:4	<1:2

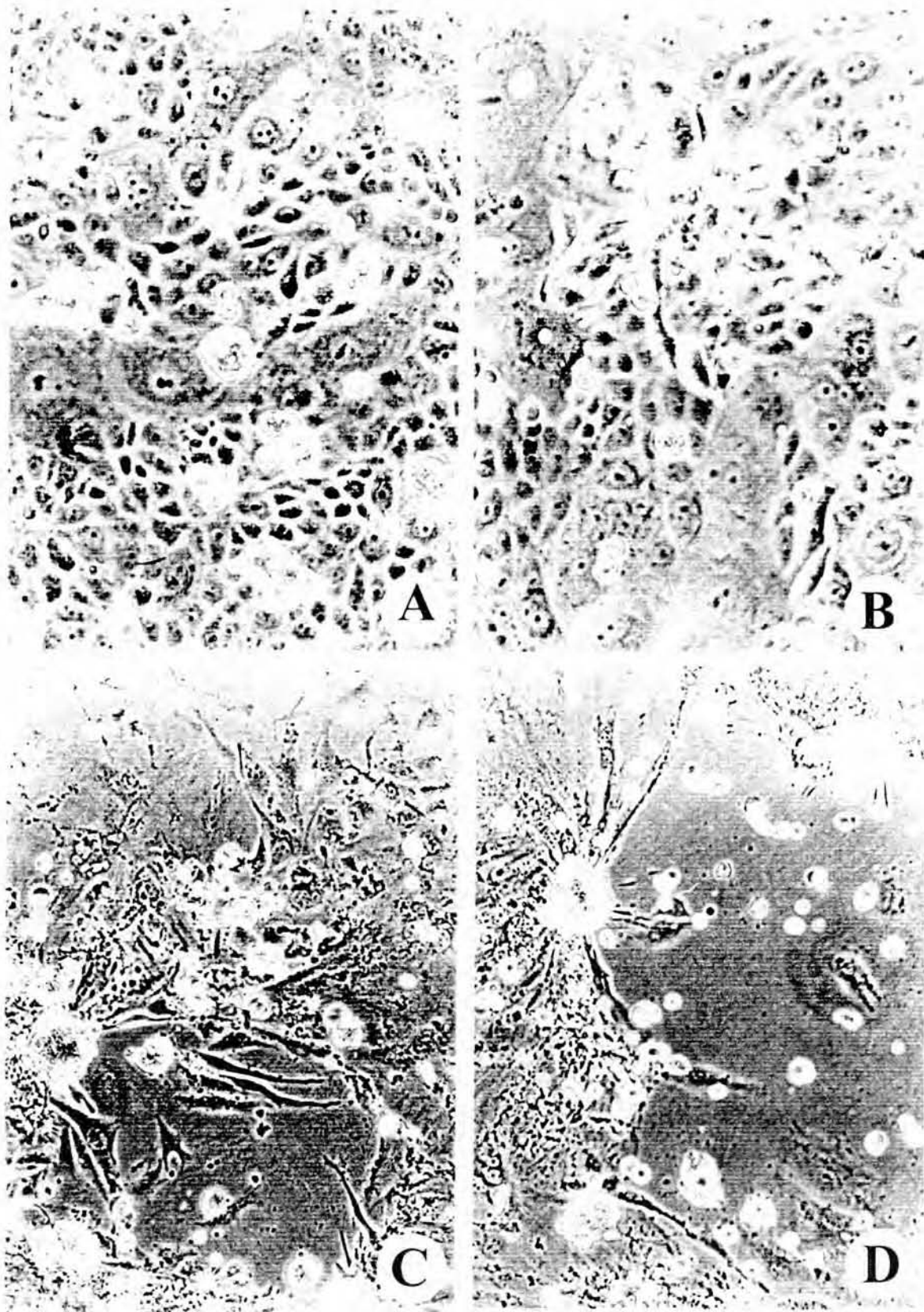


Fig. 1—Photomicrographs of non-infected cell monolayer cultures used in the studies. (A = BHK cell line; B = VERO cell line; C = CV-1 cell line; D = H9C2 cell line).

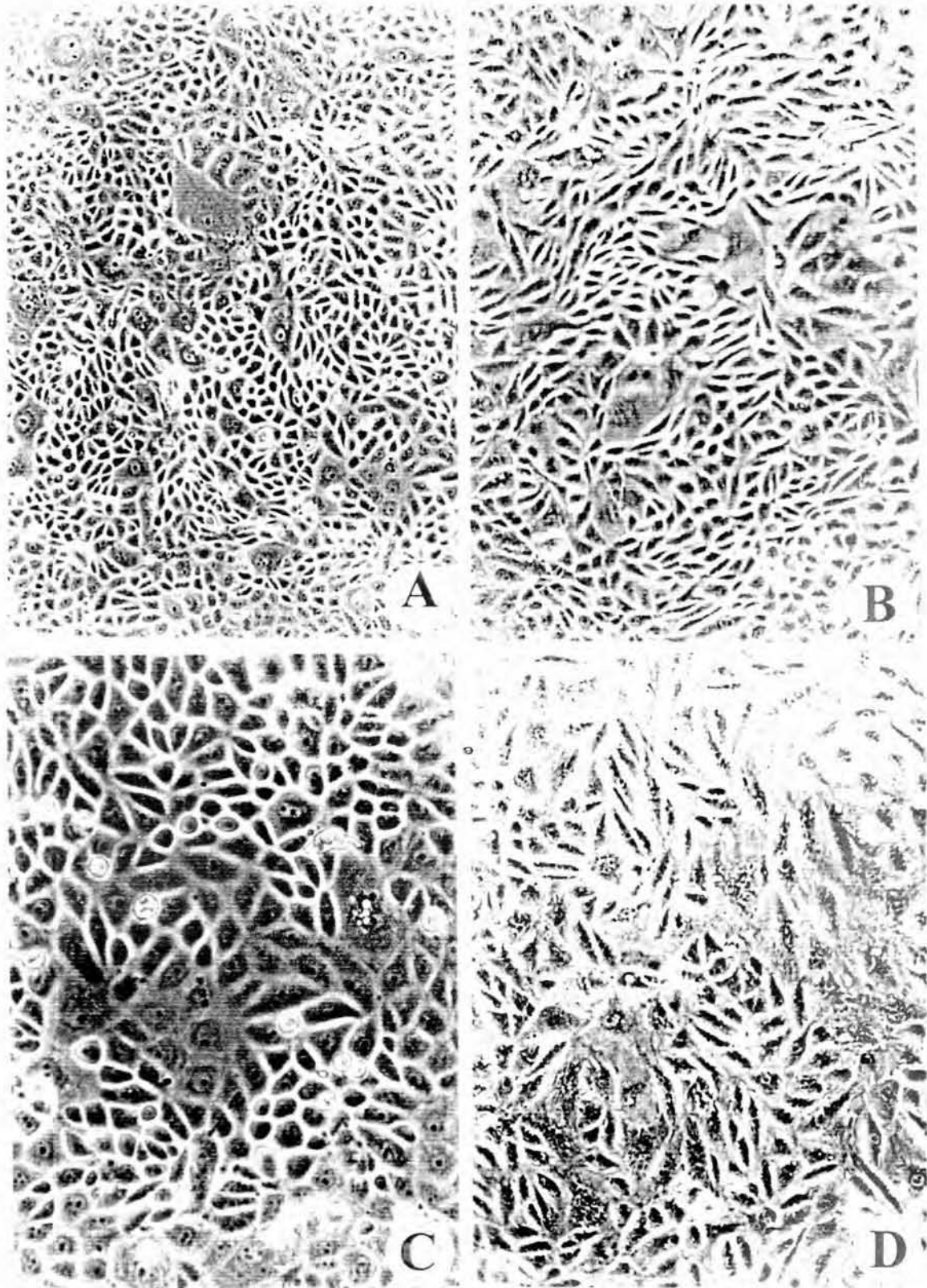


Fig. 2—Development of cytopathic effect in the case of virus-infected VERO cells. (Cells: VERO; Virus: NDV; A=15 hr post inoculation (pi); B=19 hr pi; C=22 hr pi; D=25 hr pi).

The results showed no significant difference between both the modes of the cell maintenance. In conditions of incubation within the photomultiplier chamber, the confluent monolayers of non-infected viable cell cultures used in the studies were well maintained (Fig. 2), and the CPE in virus-infected cells was properly developed (Fig. 1).

II. Registration of photon emission from non-infected and virus-infected cells

The first series of the experiments was performed using CAM cell system, which seemed to be more

probable to demonstrate the photon emission due to much higher (by order) number of cells as compared to the cell monolayer culture.

1. Photon emission from non-infected and virus-infected CAMs

Each experiment of two typical experiments, NDV being used in the 1st experiment (A) and FPV being used in the 2nd (B), show dynamics of the simultaneous registration of the photon emission from non-infected (curve I) and virus-infected (curve II) cells (Fig. 3). The parallel registration was provided by simultaneous usage of two photomultipliers,

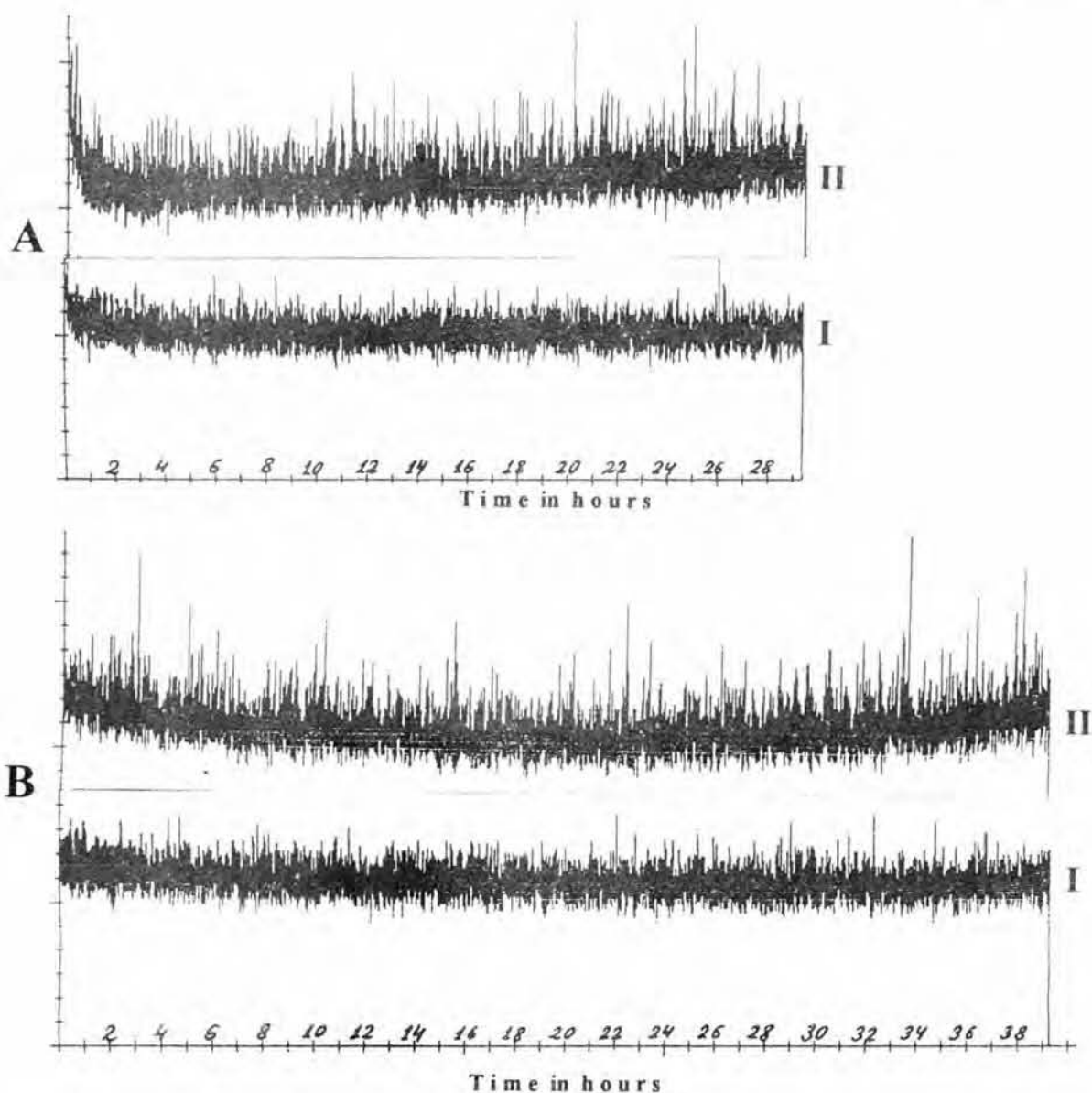


Fig. 3—Registration of photon emission from NDV- and FPV-infected chick embryo chorio-allantoic membranes (CAM) cultivated *in vitro*. (A=NDV; B=FPV; I=non-infected; II=virus-infected)

slightly differing by sensitivity that gives impression of different intensity of the measured emission in both the cases. Therefore, any comparison based on the intensity was not possible although that could be achieved by consecutive measurement of non-infected and then virus-infected cells using the same photomultiplier. However, simultaneous measurement from the non-infected and virus-infected cells using two different multipliers was preferred with the hope that more unequivocal comparison by the dynamics of emission will be more beneficent than non-simultaneous comparison of consecutive measurements. Indeed, as it can be seen, the curves corresponding to the non-infected cells, after the initial non-specific decrease (related to the instrument), show rather monotonous character with no significant changes (Fig. 3, AI & BI). Contrary to that, in the virus-infected cells, at the beginning of observation, there was a characteristic decrease of intensity (beyond the initial instrument-specific one) up to 4-5 hr post inoculation (pi) in NDV, and up to 6-8 hr pi in FPV. Then, there was increase of intensity starting from 14-15 hr up to 29.3 hr pi (the end of observation) in NDV, and from 23-24 hr up to 39.2 hr pi (the end of observation) in FPV (Fig. 3, AII & BII). Thus, in the virus-infected cells, there was definite decrease of the photon emission at the beginning of the infection, that corresponds to the initial stages of the virus multiplication cycle, and then the increase of the emission, that corresponds to virus maturation and the CPE development.

2. *Photon emission from the cell monolayer cultures*—Together with all the advantages of the

monolayer cell cultures as experimental system, there was a suspicion that the photon emission (if any) would be below any detectable level: the number of cells directly exposed to the photomultiplier's cathode is non-comparably (by order) lesser than that of any tissue object, including the above-described CAMs. Therefore, in this case, the comparison between the non-infected and virus-infected cell monolayers was certainly based not on the intensity of the emission but on any differences in the emission dynamics during the long periods of measurements. This reasoning proved to be true.

Indeed, as it can be seen in Fig. 4, the dynamics of the photon emission from the non-infected BHK cells was rather monotonous during 12 hr of measurement. Contrary to that, the respective emission from the virus-infected cells showed characteristic dynamics somewhat similar to that shown by CAM cell system with some peculiarities related to the kind of the cell cultures used for infection—BHK, VERO and CV-1 (Fig. 5). In the case of each cell culture, there was a certain decrease in the intensity at some earlier stages of the virus multiplication cycle, which was followed by a definite rise toward the end of the cycle.

In BHK cells (Fig. 5A), the decrease of the emission intensity started at 4-5 hr pi, then the rate of the decline became steeper at 9-10 hr pi, and reached its lowest point at 17 hr pi, which was followed by a definite rise observed up to 23 hr pi (the end of the measurement).

In VERO cells (Fig. 5B), after a short steep rise, there was a continuous bow-shaped decline up to 14 hr pi; this level kept on up to 16 hr pi when a definite

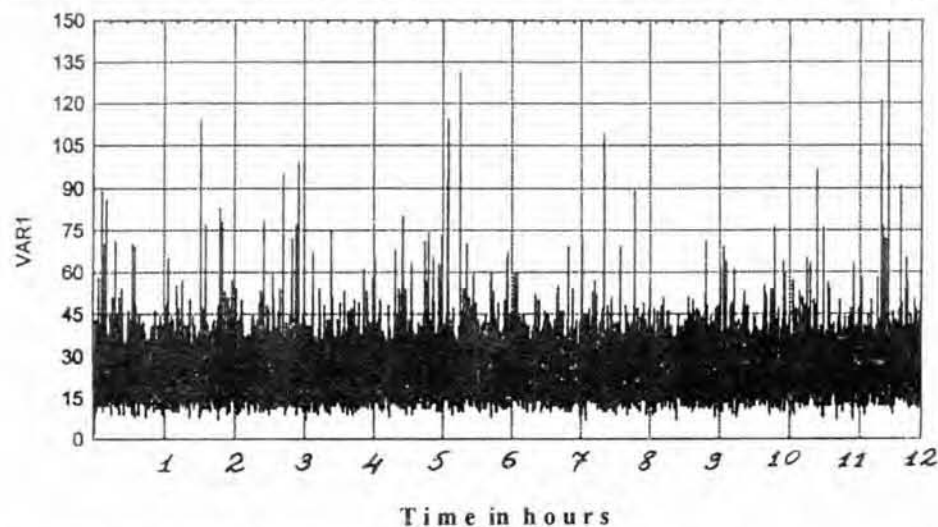


Fig. 4—Dynamics of photon emission from non-infected BHK cell monolayer culture. (Dwell time = 1 sec. Ordinate: counts per dwell time)

increase started proceeding up to 22 hr p.i., then turning into plateau level up to 25 hr. pi (the end of the measurement).

In CV-1 cell line (Fig. 5C), after a certain decrease during the 1st hr pi, the level of the emission intensity was kept on until 8.5. hr p.i., when a short rise up to the initial level kept on until about 10 hr pi, being

followed by a decline till 15 hr pi and the next rise from 18 till 23 hr p.i. (the end of the measurement).

Interestingly on the background of statistical fluctuations upon a rather smooth shape of the above rises and declines, there were a few quite distinct 'bursts' of the intensity. They occurred especially in VERO cells (the most expressed five 'bursts' at 1, 11-

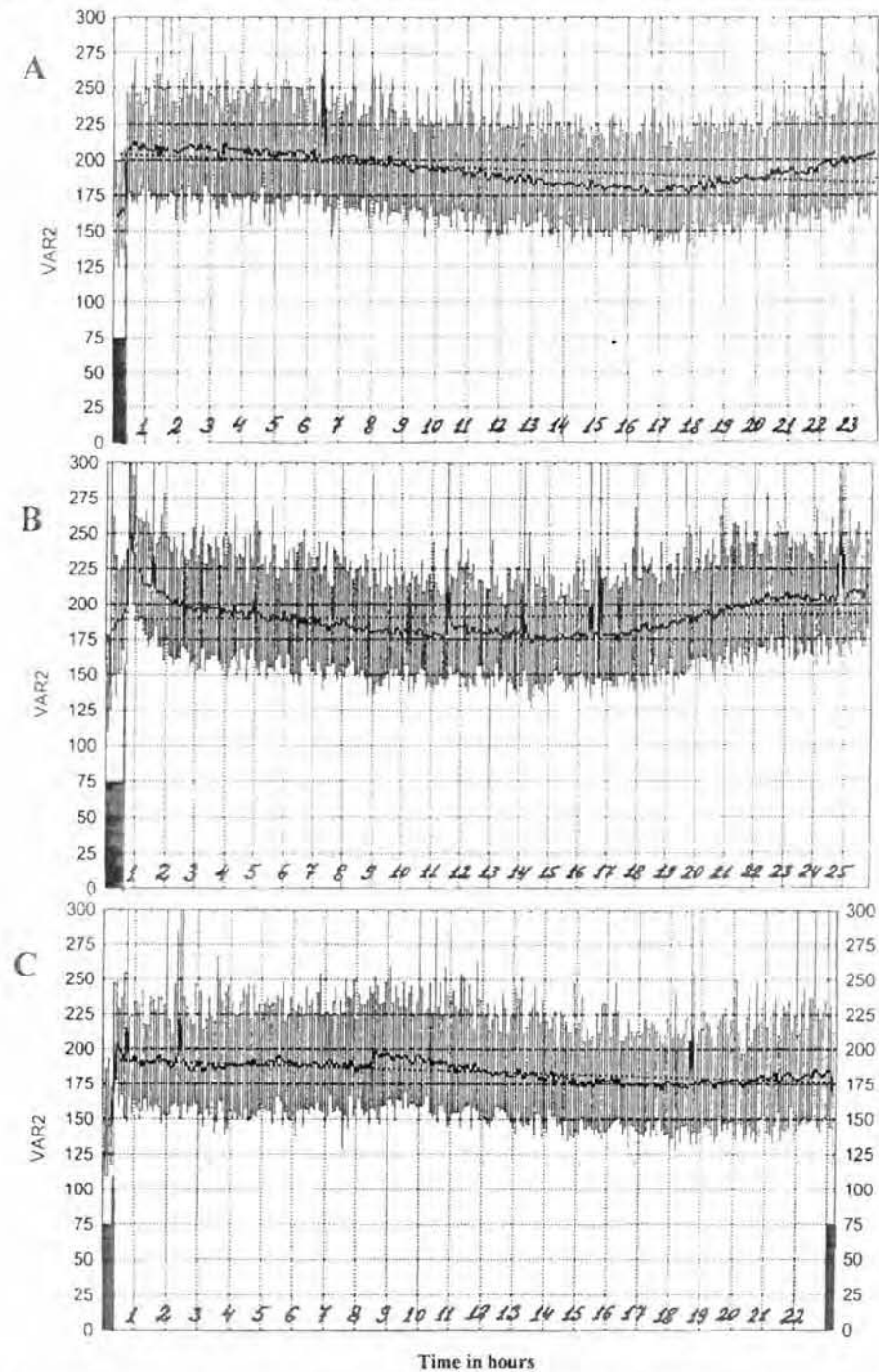


Fig. 5 — Dynamics of photon emission from virus-infected cell monolayer cultures of different origin. (A = BHK cells; B = VERO cells; C = CV-1 cells; Virus: NDV; Dwell time = 5 sec. Aggregation of the measurement values - $60 \times$ Ordinate: counts per dwell time. Blackened regions: shutters of the photomultipliers' cathodes are in closed position.

12, 16-17, and 25 hr pi, respectively), besides, three 'bursts' in CV-1 cells (1, 3, and 18 hr p.i.) and one 'burst' in BHK cells (7 hr p.i.). Since the 'bursts' evidently were not due to any artificial factors (e.g. 'external intruders'), they must be connected to certain intracellular processes occurring in virus-infected cells.

The next series of the experiments were connected with certain stages of the virus multiplication cycle, namely, (1) the very initial stage—virus penetration inside the cell, and (2) a later stage characterized by intensive CPE development.

1—In order to study photon emission during the process of virus penetration, the quartz slides with the cell monolayers were inoculated with the virus at 4°C, and after adsorption period, were put immediately into the cold maintenance medium within the quartz

cuvettes kept in the same conditions of 4°C inside the photomultiplier's chamber. The measurement procedure started at the 4°C conditions and after about 30 min, the temperature was shifted from 4° to 37°C during the continuous measurement, i.e. during course of the experiment. The non-infected cells were mimicked by the same procedure using simultaneous measurement by photomultiplier A (VAR 1). From Fig. 6, which is direct photograph documentation from the computer screen, it can be seen that just after the temperature shift, there was a very steep rise of the emission from the infected cells ('Blue') reaching the highest point 10-12 min after the temperature shift. (The process of the temperature adjustment from 4° till 37°C took about 5 min while the intensity rise started when the temperature reached 7-8°C). Then, a rather moderate decline proceeds, reaching a plateau level about half

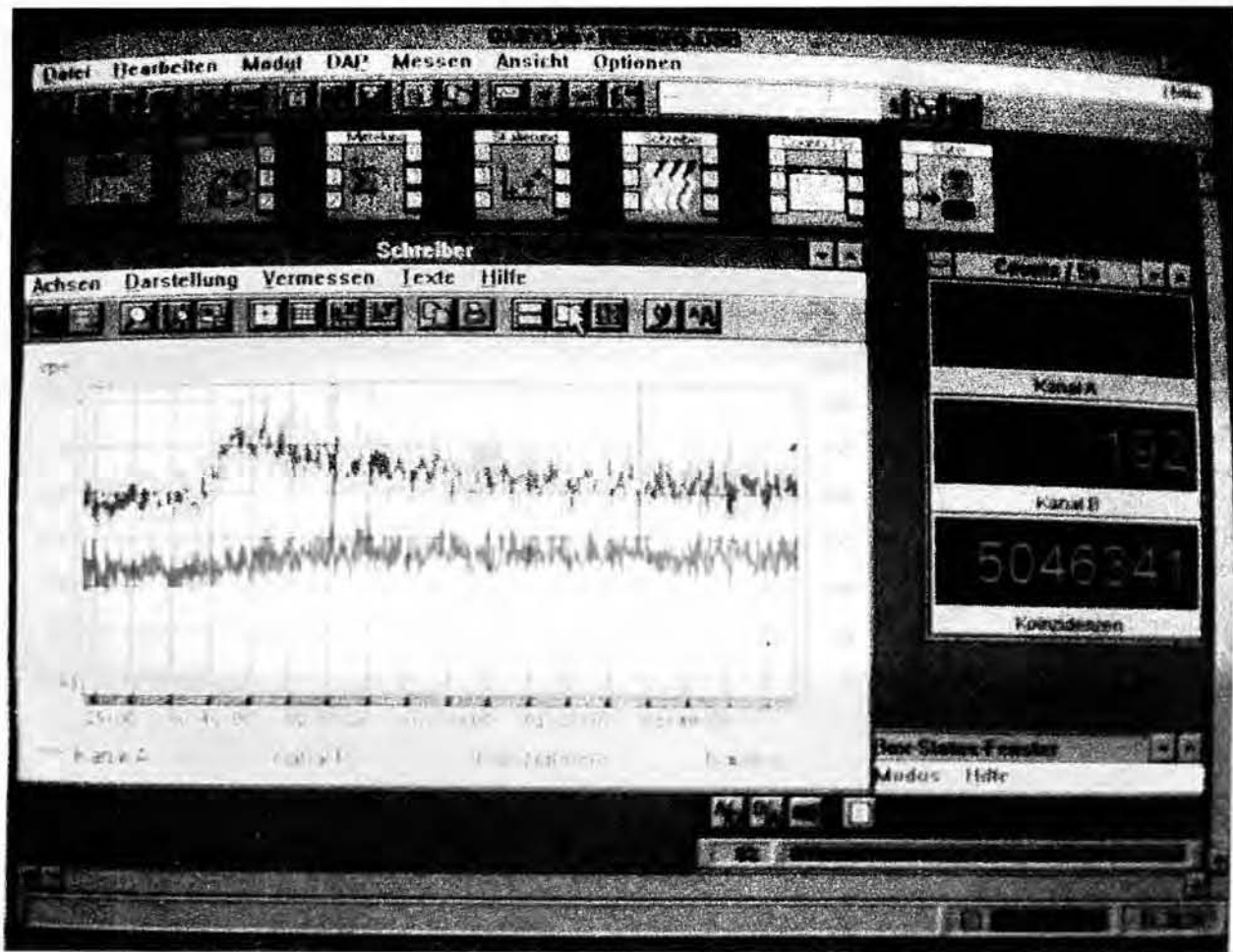


Fig. 6—Influence of temperature shift from 4° to 37°C on the dynamics of photon emission from non-infected and virus-infected cells. (Cells: VERO; Virus: NDV; Red = non-infected cells; Blue = virus-infected cells; Dwell time = 5 sec. Abscissa: time in hr and min registered from the beginning of the experiment (beyond the screen). Ordinate: counts per dwell time)

an hour after the temperature shift. The non-infected cells also react to the temperature shift but this looks like a kind of elevation to a higher level without further decline ('Red'). The experiment was repeated in different modifications, in particular, the virus-infected cells were exposed in parallel in both the photomultipliers, and a shorter period corresponding to the peak formation was analyzed in more detail (Fig. 7). The characteristic features of the above increase of the intensity were similar in both the photomultipliers (designated as VAR1 and VAR2), in spite of the difference in sensitivity. The latter was expressed in higher indications of intensity, especially in the conditions of the 37°C temperature (Fig. 7). A remarkable phenomenon was found when analyzing in detail the region of measurement during 1 hr after the start, i.e. covering the ascending region of the intensity rise: a sharp drop of the intensity as compared to the background level of the 'cold' emission (that before the temperature shift) *although the temperature started increasing*. Such 'pre-rising drop' during the temperature increase was registered by both the photomultipliers, although in the photomultiplier A (VAR1) the drop phenomenon starts about 5 min later as compared to the

photomultiplier B. This 'pre-rising drop' can become a key phenomenon for analyzing energetic grounds of the non-equilibrium molecular balance of intracellular living state (see Discussion).

2—In order to reveal the expected connection between CPE development and photon emission, the latter was studied by comparative analysis of the measurements from the (a) non-infected and virus-infected cells on the stage of the active CPE development, and (b) two virus-infected cell cultures drastically differing by their susceptibility to the virus, that determining the respective CPE development.

In accordance with the above plan, in the first experiments, two cell lines: the CV-1 susceptible to NDV and the H9C2 line of low susceptibility to this virus (i.e. causing partial CPE only after 24 hr pi, Table 1) were investigated by simultaneously performed measurements. The comparison of the virus-infected cells was carried out at the stage starting from 19 hr pi at which time there was no CPE manifestation in case of the low-susceptible H9C2 cells, while in CV-1 cell line, there was the beginning of the active CPE development. The results showed that in the H9C2 cell line, the emission intensity was

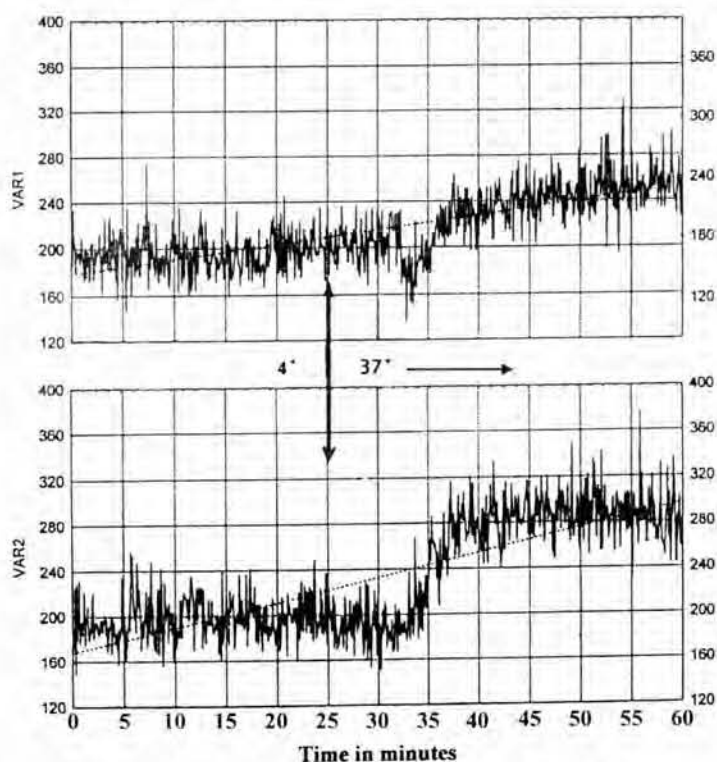


Fig. 7—Detailed dynamics of photon emission from virus-infected cells under influence of temperature shift registered simultaneously by two photomultipliers. (Cells: VERO; Virus: NDV; Dwell time – 5 sec.; Abscissa: time in minutes; Ordinate: counts per dwell time; Vertical arrow indicates the moment of temperature shift from 4° to 37°C. VAR1 = photomultiplier A; VAR2 = photomultiplier B)

rather monotonous with no difference between non-infected and virus-infected cells (Fig. 8, VAR1). Contrary to that, the virus-infected CV-I cells demonstrate non-regular burst-like rises of the emission intensity in contrast to the non-infected CV-I cells (Fig. 8, VAR2) and the virus-infected H9C2 cells (Fig. 8, VAR1).

In the other experiment, the low-susceptible H9C2 cells were compared with VERO cells—highly susceptible to NDV. The infected cells were compared at the stage starting from 27 hr pi when in H9C2 there were just initial CPE manifestations while in VERO cells the CPE development proceeded in

full swing. The results showed pretty good correlation between CPE manifestations and the photon emission intensity. Namely, in H9C2 cells, there was, although low, but significant increase of the emission from the virus-infected cells as compared to the non-infected cells, while in VERO cells, the difference between the non-infected and virus-infected cells was very well expressed (Fig. 9). Besides, in the infected H9C2 cells, there was a distinct difference between the results of the two experiments (Figs 8 and 9). Namely, the low but definite prevalence of the emission from the infected over non-infected cells at the stage of 27-28 hr pi (Fig. 9, VAR1, ' '), as compared to the full absence of any difference between non-infected and

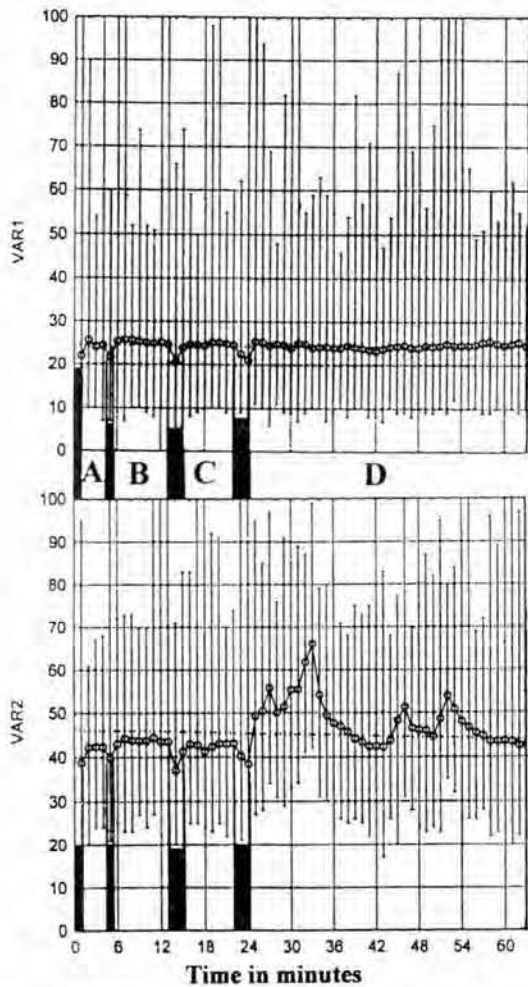


Fig. 8—Photon emission from non-infected and virus-infected H9C2 and CV-I cell cultures differing by sensitivity to virus: simultaneous measurement by two photomultipliers. (A=medium; B=quartz slide in the medium; C=non-infected cells; D=virus-infected cells exposed to measurement after 19 hr pi. Virus: NDV; VAR1: photomultiplier A: H9C2 cells; VAR2: photomultiplier B: CV-I cells; Virus: NDV; Dwell time—1 sec.; Aggregation of the measurement values—600x; Ordinate: counts per dwell time; Blackened regions: shutters of the photomultipliers' cathodes are in closed position).

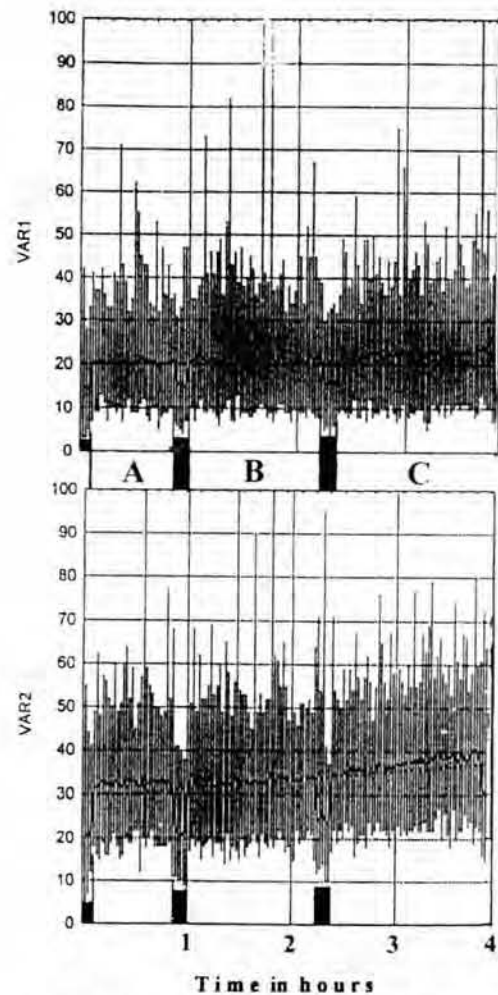


Fig. 9—Photon emission from non-infected and virus-infected H9C2 and VERO cells exposed at a later stage of the infection. (A=medium; B=non-infected cells; C=virus-infected cells exposed to measurement after 27 hr p.i.; VAR1: photomultiplier A: H9C2 cells; VAR2: photomultiplier B: VERO cells; Virus: NDV; Dwell time—1 sec.; Aggregation of the measurement values—100x; Ordinate: counts per dwell time; Blackened regions: shutters of the photomultipliers' cathodes are in closed position).

virus-infected cells at the stage of 19 hr pi (Fig. 8, VARI, D vs C) correlated with CPE development: no CPE at the 19 hr p.i. (Fig. 8D), contrary to the beginning of the CPE at stage 26-28 pi (Fig. 9C).

Discussion

The preliminary character of the described studies was due to the necessity to establish reliable experimental system permitting detection of the photon emission from the virus-infected cell monolayer cultures. Accordingly, apart from the general conditions providing an appropriate experimental system, especial elaboration concerned (a) the composition of the maintenance medium in which the cell monolayers were exposed to the photomultiplier, and (b) conditions within the photomultiplier chambers preserving normal viability of the non-infected cells, on one hand, and undisturbed virus replication in the infected cells, on the other, during prolonged period of incubation. The results have shown that no ingredient in the maintenance medium influences the measurement data as compared to the simplest PBS buffer solution, and the prolonged incubation within the photomultiplier chambers does not influence viability of the cell cultures and the development of the virus-induced CPE.

In the main experiments related to registration of the photon emission from the virus-infected cells, two questions had to be elucidated: (1) the scientific question whether at all specific process(es) within the virus-infected cells cause the photon emission, and (2) the technical question whether the intensity of the presupposed photon emission from the virus-infected cell monolayer culture containing small quantity of cellular bio-mass per sample can be detected by the existing equipment. However, namely the latter question determining a general suitability of the developed experimental system was the main topic of the presented studies.

In this respect, the absence of significant prevalence of the measurement intensity in non-infected cultured cells versus nutritive medium (Fig. 8 B vs. A and Fig. 9 B vs. A) would look disappointing as if showing the absence of (or at least impossibility to detect) any actual photon emission from the cultured cell system. However, the experiments on the CAM system containing a huge number of cells appeared to be an indicating system by demonstrating a clear difference between the non-infected and virus-

infected CAMs, expressed by the monotonous character related to the non-infected CAMs versus specifically distinctive dynamics related to the virus-infected CAMs (Fig. 3). A similar picture, in principle, was obtained in cell monolayer cultures (Figs 4 and 5) containing considerably lesser (by order) number of cells exposed to the photomultiplier. Such a combination as itself is (although indirect) a proof of the photon emission from *both* the virus-infected and non-infected cells. If in this combination the non-infected cells are accounted as a 'control', the observed monotonous dynamics impart to them an optimal quality – stability, which is desirable for any 'control' object, but here such 'control' does not mean just the absence of emission. The *decline* in the measurements dynamics in the virus-infected cells as compared to the monotonous dynamics in the non-infected cells should be considered as a proof of a certain 'activity state' in the virus-infected cells. The decrease of anything may occur if the respective 'something' exists. If that 'activity state' is guessed in virus-infected cells, such 'state' must emerge from an initially existing 'normal activity state' of the non-infected cells. A more detailed analysis showed more evident displays of the photon emission in virus-infected cells as compared to the non-infected ones, in particular, at the stage of virus penetration inside the cells (Fig. 8) and a correlation between the photon emission and the CPE manifestations (Fig. 9).

Thus, these presented 'technical' results have prepared potential basis for the studies on the scientific question, namely, which, if any, specific process(es) within the virus-infected and non-infected cells cause the photon emission. The research in this respect must be based on much more sophisticated statistical analysis of the emission regime suggested to depend on highly complicated combinations of different rhythms associated with intrinsic living processes. In this respect, the advantage of the employment of the virus-infected cells is associated with the *irreversibility* of the intracellular processes in these cells, that being associated with virus replication, CPE development, and, finally, cell death. The latter has been recently shown to occur by apoptotic mechanism, i.e. 'programmed cell death'²⁵⁻³¹. In full contrast to that, the non-infected cells derived from the cloned stable cell line are expected to demonstrate a more homogeneous rhythmic regime of cyclical recurrence of the emission parameters, that would be compatible with a state of non-equilibrium orderliness in living systems^{4,5,7,8}.

Meanwhile, the only phenomenon which may be considered and analyzed as a signal from the intracellular 'core' in the infected cells is that one found in the experiments dealing with the temperature shifts (Fig. 7). Namely, as a result of the temperature shift from 4° to 37°C, there is synchronous penetration inside the cells of all the viral particles pre-adsorbed upon the cell surface at the 4°C. Just after the temperature shift, however, there was a paradoxical sharp drop of the intensity before its 'ordinary' increase (i.e. that expected from the generally suggested reasons—dependence of the intensity of intracellular metabolic processes on temperature). This 'pre-rising drop' may be also explained by the A. Gurwitsch's idea about non-equilibrium (non-balanced) molecular constellations as an intrinsic quality of any living^{4,5}. The possibility of the existence and maintenance of such constellations has been explained by the theory of biological field^{7,8,10,11}.

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