of the 5 ml-fractions and the biological activity of the pooled fractions are shown in Figure 3. It is evident that the clearly separated peaks Nos. 2 and 3 showed the expected inhibitory activity. The molecular weights, according to the K_{av}-values, are in the order of 30,000 daltons and 11,000 daltons for peak 2 and 3 respectively.

After a number of trials the scheme shown in Figure 4 proved to be a workable basis for the purification of the inhibitory compound. The fractionation together with the biological activity of the Sephadex G-75 is shown in Figure 5. On the basis of the determined K_{av}-values, the molecular weights of the active compounds could again be calculated as 28,000 and 11,000 daltons respectively. Electrofocusing of the 2 active peaks from G-75 filtration showed that these peaks do not represent pure compounds. The results are shown in Figure 6. When divided up into the individual peaks and percolation over G-25 (for removal of sucrose and ampholines) as obtained after electrofocussing, the biological activity was lost. No attempt at this stage was made to pool all the electrofocussed peaks and to try if biological activity could thus be restored.

Peaks Nos. 2 and 3 from Sephadex G-75 fractionation were subjected to SDS-polyacrylamide gel electrophoresis to estimate the degree of purity and possible sub-unit character of the coumponds. 2 stainable bands could be detected with apparent molecular weights of the compounds of 27,000 and 12,000 daltons respectively. The tests on differential absorbance at 260 and 280 nm of these peaks also showed that the active principles are proteins; according to this method virtually no nucleic acids could be detected. It would seem premature to present speculations on the mechanism of action of these proteins. Experiments along this line are in progress.

Zusammenfassung. Mittels Ultrafiltration und Gelelektrophorese konnten 2 extrazellulare Protein-Fraktionen, Mol.- Gew. 27000–28000 daltons und 11000–12000 daltons, aus submersen Kulturen von *Aspergillus oryzae* gewonnen werden, welche Wachstumshemmung desselben Organismus bei Verabreichung zu Beginn der Kulturentwicklung verursachen.

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Herpes-Virus and Double-Stranded RNA

Although 14 years have passed since the discovery of interferon, the exact nature of its induction by viruses is not yet completely understood.

It appears that RNA needs a well defined secondary structure to be a good interferon inducer, true double-stranded ribonucleic acids being better inducers than single-stranded forms[1–7]. However, DNA appears to be a very poor inducer of interferon, if it works at all. The question of what the inducer nucleic acid is in DNA viruses needs to be explored further.

The report of Colby and Duesberg[8] that vaccinia virus induces a double-stranded (DS) RNA in chick embryo cells offered a possible explanation of the phenomenon. However, the necessity for DS RNA during replication of DNA viruses has not been shown.

Herpes simplex virus displays a variable pattern with regard to interferon induction; the same viral strain, in the same non-permissive cells, at a low multiplicity of infection (MOI) induces interferon production, while no viral constituents are synthesized; at a higher MOI, viral constituents are produced in a defective manner, but no detectable amounts of interferon are present[9].

If a DS RNA is a necessary step during replication of DNA viruses, one can assume that interferon appears when the virus-induced DS RNA occurs in a cell population with conserved protein biosynthesis. The present communication deals with our attempts to check such a hypothesis.

Materials. All experiments were carried out on primary chick embryo cells (CEC) and on HEp-2, MA-104 and mKS-B[10] cell lines. *Herpes simplex* virus, strain S, (a gift from Dr. G. Tarro), vesicular stomatitis virus (VSV) and vaccinia virus were replicated on Hep-2 cells. Titers: *Herpes simplex* virus, 3×10^8 PFU/ml; VSV, 5×10^6 PFU/ml; vaccinia virus, 5×10^8 $TCID_{50}$/ml on HEp-2 cells.

Results and discussion. Monolayers of barely confluent CEC were infected with *Herpes simplex* virus (MOI 10 PFU/cell), a second set of replicates was infected with vaccinia virus (MOI 10 PFU/cell), while a mock-infected group of cultures served as control. After an adsorption period of 1 h, nutrient medium (without serum) containing 5 μCi/ml of uridine-5-^{3}H was added and the cultures were incubated at 37 °C for 6 h, since it is well known that at this moment all RNA synthesis is herpes-directed[11–12].

In other series of experiments, cultures were incubated for 18 and 24 h after infection, and extraction of RNAs was performed as described by Colby and Duesberg[8].

Under identical conditions this experiment was repeated with HEp-2, mKS-B and MA-104 cell cultures with similar results. 1. LiCl precipitation of RNAs from *Herpes*-infected and uninfected cells was performed as

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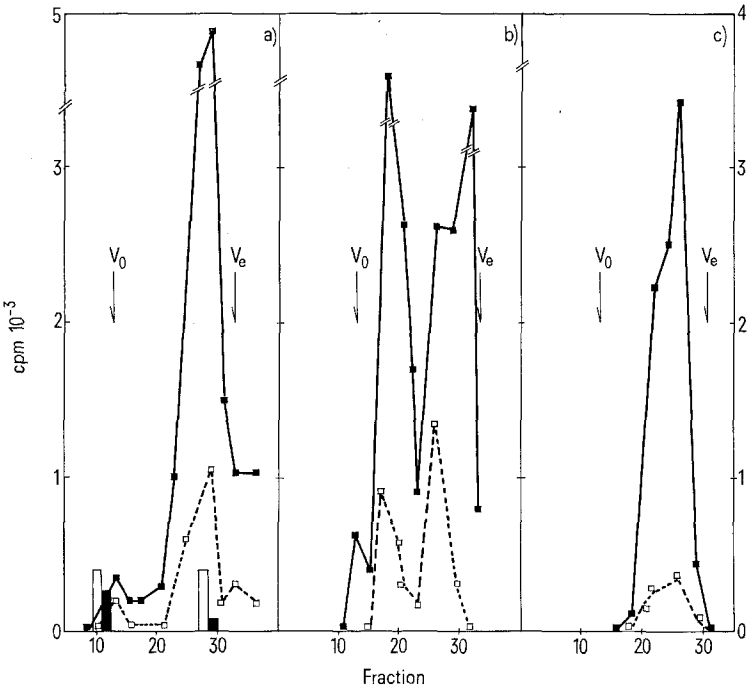

Fig. 1. Gel filtration in a Sephadex G-200 column of the soluble fraction or RNA after 2 *M* LiCl precipitation. Continuous line, TCA-precipitable radioactivity; dotted line, TCA-precipitable radioactivity after RNase treatment; arrows indicate the void volume (Vo) and the elution volume of phenol red (Ve). a) Vaccinia infected CEC, b) herpes infected CEC, uninfected CEC.

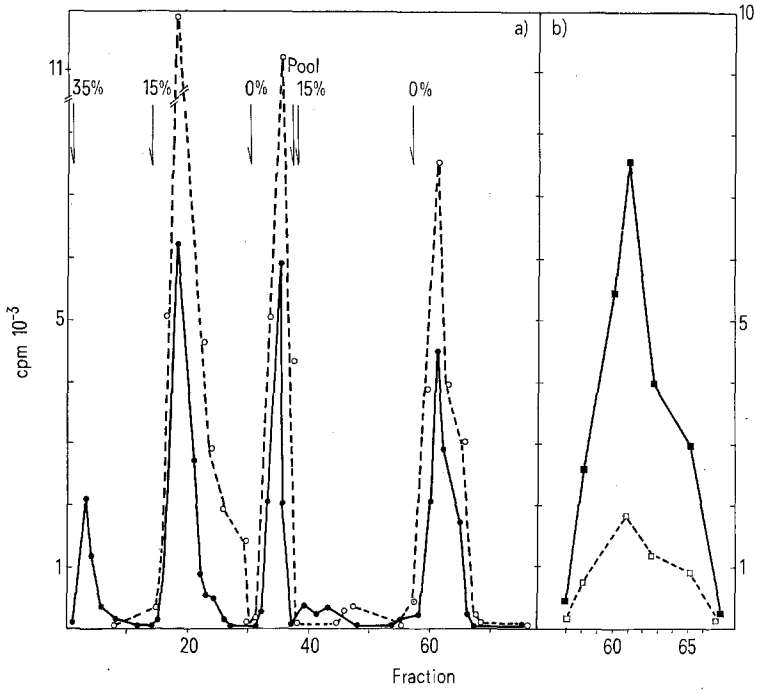

Fig. 2. Cellulose chromatography of herpes directed RNA. a) 8 O.D.$_{260}$ of RNA from herpes infected cells in a total volume of 0.9 ml of buffer (Tris-HCl 50 m*M*, pH 6.9; NaCl 100 m*M*; EDTA 1 m*M*) equilibrated with ethanol (65/35 v/v) were inoculated on the top of a cellulose column; a mixture of buffer-ethanol (65/35) was pumped and when O.D. record came back to the base line and no more radioactivity was detected in the effluent, the eluent was made 15% ethanol (buffer-ethanol 85/15 v/v). A second peak appeared and chromatography was continuated until extinction of O.D. and exhaustion of radioactivity. Then the buffer alone was pumped through the column and the 3rd peak could be recovered. Continuous line, O.D.$_{254}$; dotted line, TCA-precipitable radioactivity. b) The fractions of the 3rd peak were analysed for RNase resistance. Continuous line, TCA-precipitable radioactivity; dotted line, TCA-precipitable radioactivity after RNase treatment.

described by FALCOFF and FALCOFF[13]. It is expected that, after LiCl precipitation, the soluble fraction will contain both low molecular weight sRNA and any DS RNA present. If indeed both these components are present, gel filtration of the soluble fraction may be a reasonable tool to separate them.

When such an experiment was performed with RNA from vaccinia virus infected cells, a little peak of radioactivity partially resistant to RNase hydrolysis (60% in 2 × SSC) was eluted in the void volume, while neither in *Herpes*-infected nor in control cells was a similar RNA present (Figure 1a, b, c). 2. Zonal centrifugation: The soluble and insoluble fractions after LiCl precipitation were centrifuged through a density gradient of sucrose 5–20% in acetate buffer. TCA-precipitable radioactivity and RNase resistance were determined. Once more, no difference was found between *Herpes*-infected and control cells. 3. Cellulose chromatography: Fractionation of RNAs was accomplished by step-wise elution from a cellulose column as described by FRANKLIN[14].

As shown in Figure 2a and 2b, RNase treatment diminished by more than 70% the TCA-precipitable radioactivity present in the fractions of the 3rd peak, both in *Herpes*-infected and in uninfected cells. We concluded that the 3rd peak was only a persistant contaminant rRNA, despite 3 washings with ethanol 15%. This conclusion was confirmed by measuring the distribution of radioactivity after a sucrose density gradient centrifuge run of the pooled fractions of the third peak. 4. Induction of interferon: It has been suggested that interferon induction may be considered as a biological marker of DS RNAs[15].

RNAs of both *Herpes*-infected and uninfected cells were inoculated i.p. in adult Swiss mice (200 µg/mouse) and after 5 h sera were collected and assayed for interferon by plaque reduction method in L-cells. No detectable amounts of interferon were found. In order to test the ability of *Herpes*-directed RNA to induce interferon production in vitro, 2-day-old monolayers CCL1 cells were pre-treated with DEAE-dextran, 100 µg/ml, 2 h, 37 °C. Then cell sheets were washed twice with nutrient medium and incubated overnight at 37 °C with RNAs from *Herpes*-infected and mock infected CEC, HEp-2, MA-104 and mKS-B cultures. Cultures treated with poly 1:C (10 µg/ml) were included as a positive control.

After the overnight incubation period, nutrient media were removed, the cells were washed twice and infected with VSV (MOI = 5 PFU/cell). After 18 h incubation at 37 °C, nutrient fluids were harvested and emergent virus was titrated. As shown in the Table, poly 1:C diminished by about 1000-fold the yield of the virus, while *Herpes*-directed RNAs were not able to reduce the replication of the challenge virus.

Vaccinia virus-induced, double-stranded RNA was characterized by 2 main criteria: nuclease resistance and gel filtration exclusion[8]. Very likely the vaccinia virus-induced material we found in the exclusion volume of the G-200 Sephadex column after LiCl precipitation (Figure 1) is the same as described by COLBY and DUESBERG[8], although it is less resistant to RNase hydrolysis (60% vs. 97%), but probably the difference in the preparative method may be responsible for the diminished RNase-resistance; LiCl precipitation is obviously a milder method as compared with nuclease digestion prior to gel filtration and it cannot be excluded that contaminant single-stranded RNA could be present in our preparation. We were unable to detect a similar material in RNA from *Herpes*-infected cells.

Density gradient separation and cellulose chromatography additional support to the conclusion that DS RNA is not found in *Herpes*-infected cells. It is obviously impossible to exclude the possibility that such RNA might exist in minimal amounts, far below the levels of detection of the biochemical methods employed. But a further indication of its absence in *Herpes*-infected cells came from biological tests.

Monolayers of CCL1 cells were pretreated with DEAE-dextran (100 µg/ml, 1 h at 37 °C). After washing twice, cultures were overnight incubated with inducers at 37 °C, washed twice again and infected with 5 PFU/cell of VSV. Emergent virus was titrated on HEp-2 cells.

Virus yield of CCl cells treated with RNA from herpes infected and mock infected cells

Pre-treatment	Inducer	Emergent-VSV
—	—	1.1×10^5
DEAE-dextran	—	7.0×10^5
DEAE-dextran	Poly I:C	4.5×10^2
DEAE-dextran	RNA (HEp2)	1.3×10^5
DEAE-dextran	RNA (HE2/Herpes)	6.0×10^5
DEAE-dextran	RNA (mKS-B)	1.0×10^5
DEAE-dextran	RNA (mKS-B/Herpes)	6.1×10^5
DEAE-dextran	RNA (MA-104)	1.1×10^5
DEAE-dextran	RNA (MA-104/Herpes)	1.1×10^5

Since interferon induction is a very sensitive biological marker of DS RNA (even if not all DS RNA are good interferon inducers) 2 suitable systems were assayed: CCL1 cells and mice. As reported here *Herpes* virus-directed RNA was unable to induce interferon production either in vitro or in vivo. The role of the host was also taken into account and different cell systems were tested; it could be supposed that DS RNA appears only in interferon producer systems. CEC, mKS-B and MA-104 cells are well known producers of interferon and it has been reported that double-stranded polyribonucleotides induce an antiviral resistant state in HEp-2 cells[16]. The importance of the host, however, seems not to be critical, since DS RNA should be a virus-directed event, independent, or at least partially independent, of the host cell.

The uniqueness of vaccinia virus must also be kept in mind: its structure, behaviour and its replication site are completely different from those of other DNA viruses. The presence of a DNA-dependent RNA polymerase included in the virion (and essential for its integrity) perhaps plays a crucial role in the mechanism of appearence of DS RNA: transcription of complementary strands might be only a result of a less accurate recognition of initiation sites on the template.

Recent reports by BAKAY and BURKE[17], together with ours in the *Herpes* system and our unreported observations with the cytoplasmic polyhedral DNA virus FV3 (manuscript in preparation), makes the assumption very unlikely that DS RNA might be a phenomenon of general validity for all DNA viruses.

When this manuscript was in preparation, LUCAS and GINSBERG[18] reported the identification of a virus-coded, DS RNA in KB cells infected with type 2-adenovirus.

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Résumé. On a cherché à déterminer la presence d'un ARN bi-caténaire lors de la réplication du virus herpétique. La précipitation de LiCl 2 *M*, la filtration sur gel Sephadex, la chromatographie sur cellulose, la sédimentation en gradient de densité et l'induction d'interféron in vivo et in vitro semblent démontrer qu'une telle structure n'est pas indispensable à la réplication du virus herpétique.

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PRO EXPERIMENTIS

Radiological Determination of Heart Volume in Rats

In a preliminary study on experimentally induced cardiac hypertrophy and regression of the hypertrophy in rats, we found it necessary to develop a method for in vivo estimation of the heart volume. Our aim was to obtain information on changes in the heart volume of the same animal at different stages of the experiment. The method by JONSELL[1] for estimation of the heart volume in human beings was modified for use in rats. This paper is a report on the method and the changes of the heart volume relative to body weight in growing rats.

Animals and methods. Albino Wistar male rats aged 16 (group A) and 24 weeks (group B) were used. Each group consisted of 10 rats. The animals were reared on a commercial pelleted diet (Hankkija). The rats in group A were followed up to the age of 20 weeks, and in group B to the age of 35 weeks. After initial determination of the heart volume and body weight, these parameters were determined 5 times in group A and 4 times in group B at the time intervals indicated in the Table. During the study 2 rats in group A and 1 in group B died. The radiographs for determination of the heart volume were taken under light ether anaesthaesia with the rats fixed with adhesive tape to a plate especially designed for this purpose (Figure 1). At each determination 3 pairs of frontal and lateral films were made. The heart volume was expressed as the mean value of the volumes calculated from those films. The frontal and lateral films were exposed with the same values: 55 kV, 500 mA and 3 msec at a focus-film distance of 60 cm. The X-ray tube used had a focus size of 1.2 × 1.2 mm². The pictures were taken on an ordinary X-ray film for 90 s processing, and high resolution screens were used in the casette. Equipment for simultaneous exposure of the frontal and lateral projections was not available.

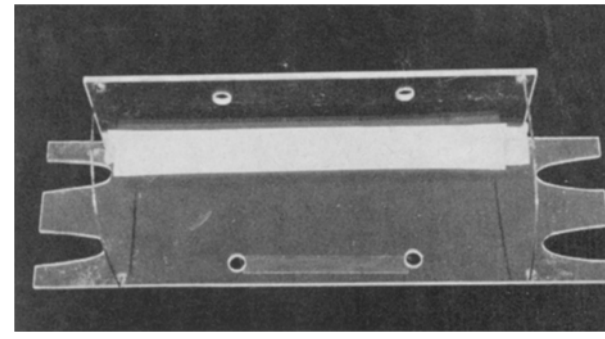

Fig. 1. Plate made of plexiglass on which the rats were fixed during picture-taking.

For determination of the heart volume, the rat heart was assumed to be an ellipsoid. Thus, the formula $V = l \times b \times d \times k$ generally used for determination of the human heart volume[1] could be used. In this formula l (length) and b (breadh) were measured from the frontal film and d (depth) from the lateral one (Figure 2). The constant $k = 0.47$ was calculated from the formula for the volume of an ellipsoid $3\ \pi/4\ {}^{l}/_{2} \times {}^{b}/_{2} \times {}^{d}_{2}$. Allowance was made for the geometrical enlargement due to the ratio of the focus-film and heart-film distances.

Results. The heart volumes of the rats in both groups are given in Figure 3. The deviations of the individual heart volumes from the growth curve increased with age. From 6 to 35 weeks the standard deviations of the heart volumes from the means increased from 245 to 655 mm³. Despite these deviations, the individual heart volume growth patterns were very similar, indicating that the deviations did not result from differences in volume determinations. Up to the age of 15 weeks, there was a statistically significant increase ($p < 0.001$) in the heart volumes between the given time intervals. After this age, a non-significant increase was noted up to 35 weeks of age.

Heart volume/body weight ratios of growing rats

Group	No. of animals	Age (weeks)	Heart volume/body weight ratio	
			mm³/g	*P*
A)	10	6	10.0 ± 0.9[a]	
	9	8	9.2 ± 0.9	NS[b]
	9	10	9.5 ± 1.1	NS
	8	15	8.4 ± 0.6	<0.005[b]
	8	20	7.7 ± 0.4	<0.05[b]
B)	10	24	8.6 ± 0.6	NS[c]
	9	27	9.0 ± 1.1	NS[c]
	9	31	8.8 ± 0.9	NS[c]
	9	35	8.7 ± 0.5	NS[c]

[a] Mean ± S.D. [b] Compared to group A, 6 weeks. [c] Compared to group A, 15 weeks. The same rats as in Figure 3.

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