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PROPERTIES OF AN INFECTIOUS NUCLEIC ACID FRACTION FROM CHICKEN EMBRYOS WHICH HAVE BEEN INFECTED WITH ENCEPHALITIS VIRUS

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Second Communication: Biological Properties

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Received on 5 November 1959.

The multiplication of normal Eastern-Equine-Encephalomyelitis (EEE) viruses is stimulated in the incubated, fertilized chicken egg by means of nucleic acid fractions which were obtained from infected chicken embryos. Plaques can be produced in tissue cultures from embryonal chicken cells with nucleic acid fractions. The infectiousness of the nucleic acid fractions is subject to various inhibitory influences due to substances which are contained in them, in addition to the infectious, high-molecular RNA. These inhibitors can be partly removed or inactivated by means of alcohol precipitation of RNA and by a treatment with desoxyribonuclease. The infectious RNA in the nucleic acid fractions does not come from the EEE virus elementary particles but rather from a ribonuclease-sensitive virus-specific material which must be present in the infected cells.

In an earlier communication (/1/ E. Wecker, Z. Naturforschg. 14b, 370, 1959) we reported on the physical and chemical properties of infectious nucleic acid (NA) fractions which can be isolated from chicken embryos that have been infected with the virus of American Equine Encephalomyelitis type East (EEE virus).

This second communication here today deals with the biological properties of such NA fractions.

The infectiousness of RNA preparations, which could be obtained from

various types of viruses according to the phenol method of Gierer and Schramm (/2/ A. Gierer and G. Schramm, Nature, London, 177, 702, 1956; Z. Naturforschg. 11b, 138, 1956), usually is about 0.1-1% of that of the original virus preparations (/2/), (/3/ H. E. Alexander, G. Koch, J. M. Mountain, K. Sprunt and O. van Damme, Virology 5, 172, 1958), (/4/ R. M. Franklin, E. Wecker and C. Henry, Virology 7, 220, 1959), (/5/ J. S. Colter, H. H. Bird and R. A. Brown, Nature, London, 179, 859, 1957), (/6/ J. S. Colter, H. H. Bird, A. W. Moyer and R. A. Brown, Virology 4, 522, 1957). In all of these cases, the infectious RNA certainly (/2-4/) or at least most probably (/5, 6/) (/7/ F. Brown, R. F. Selters and D. L. Stewart, Nature, London, 182, 535, 1958) from the particular elementary virus particles themselves. Later on, Huppert and Sanders (/8/ J. Huppert and F. K. Sanders, Nature, London, 182, 515, 1958), as well as Brown and Stewart (/9/ F. Brown and D. L. Stewart, Virology 7, 408, 1959) reported on even greater differences between the infectiousness of virus preparations and of RNA preparations. But these authors indicated the probability that their RNA preparations were not extracted from the particular elementary virus particles themselves but rather from a different virus-specific material.

The NA fractions from EEE virus-infected chicken embryos described here have an average of about only 0.1 permill of the infectiousness of the corresponding virus preparations. We had arrived at similar results in experiments in which the NA fractions had been prepared from infected mouse brains (/10/ E. Wecker and W. Schafer, Z. Naturforschg, 12b, 415, 1957). Let us now examine and discuss some of the reasons for the relatively low infectiousness of our NA fractions. (The results of these investigations were reported by E. Wecker at the 4th international congress of biochemistry in Vienna, 1958; (Pergamon Press, London, New York, Paris, Los Angeles) and W. Schafer at the symposium on "virus growth and variation," London 1959; University Press, Cambridge.).

Materials and Methods

1. Virus Preparations

(a) EEE virus (The EEE and WEE strains were made available to us through the kindness of Prof. Dr. E. Traub, West German Federal Research Institute for Animal Virus Diseases, Tübingen): 11-12-day old chicken embryos, which were infected with about 10 egg-LD_{50}, were frozen wherever possible right after death at -40° C. Each time, a frozen embryo was homogenized with 3 ml of 0.02-m. phosphate buffer, with a p_H of 7.0, for a period of 5 minutes, under ice-cooling /refrigeration/ and the homogenate was then centrifuged for 10 minutes at 3,000 rpm. The supernate then represented the EEE virus preparation.

(b) Western-Equine-Encephalomyelitis (WEE) virus: Tissue cultures from embryonal chicken cells were placed in Petri dishes. The medium consisted of Earle's solution which contained 0.5% lactalbumin-hydrolysate, 0.1% yeast extract, and 10% calf serum. One day after the inoculation of the cells, the plaques were infected with about 10^7 plaque-forming units (PBE). When

the cells reveal a pronounced cytopathogenic effect (mostly after about 24 hours), the medium was removed and frozen after the centrifugation of the remaining cell components at -40° C. From a total of 2 liters of such WEE-containing media, a purified virus concentrate was produced by means of several cycles of high-rate and low-rate centrifugation as well as by treatment with ribonuclease and desoxyribonuclease.

2. Isolation of NA Fractions

The NA fractions are obtained from infected and normal chicken embryos as described in communication I (/1/).

3. Infection Tests

(a) In the Incubated, Fertilized Chicken Egg

In each case, we take 0.2 ml of the NA fraction, respectively, virus dilution in 0.1-m. phosphate buffer (p_H 7.5) and we inject them into the amnionic cavity of chicken eggs that have been incubated for 11 days. For each dilution, we used 10 eggs; the dilutions were made by powers of 5 or 10. The titer was determined according to Reed and Muench (/11/ L. J. Reed and H. Muench, Amer. J. Hyg. 27, 493, 1938).

(b) In the Plaque-Test

The plaque tests according to Dulbecco (/12/ R. Dulbecco, Proc. nat. Sci. USA 38, 747, 1952) were performed in the manner described earlier for the virus of classical /conventional/ fowl pest, on tissue cultures of embryonal chicken cells (/13/ E. Wecker and W. Schafer, Z. Naturforschg. 11b, 181, 1956).

4. Serums

(We would like to express our thanks to Prof. Dr. E. Traub, Tübingen, Prof. Dr. W. Henle, Philadelphia, Dr. M. Mussgay, Tübingen, Dr. W. McD. Hammon, Pittsburgh and Dr. H. R. Cox, Lederle Lab. for their kindness in letting us have more EEE antisera.)

In the case of the EEE antiserum used here we are dealing with a rabbit serum against the "Princeton" strain with a specified neutralization index of 0.5 log. We obtained this serum from the Department of Public Health, Berkeley, California.

5. Production of Gamma-Globulin Fractions

(I want to thank Dr. H. D. Matheka, West German Federal Research Institute for Animal Virus Diseases, Tübingen, for familiarizing me with and briefing me on this method.)

The globulins are precipitated by means of the 50-% saturation of the particular serum with ammoniumsulfate. The centrifuged precipitates were dissolved in distilled water and were then dialyzed for 2 days against water whose p_H was adjusted to about 7. The finished dialysates were then diluted with water down to a total protein concentration of about 1% and the beta globulins were then precipitated isoelectrically at a p_H of 4.9. After the centrifugation of the precipitates, the supernates were again neutralized with NaOH, they were reduced to the original serum volume by means of evaporation in the dialysis tube, and finally NaCl was added up to the physiological concentration.

6. Effect of Sera on NA Fractions or Virus Preparations

The virus preparations or NA fractions were diluted in 0.1-m. phosphate buffer (p_H 7.5) by powers of 10. To each dilution we then added an equal volume of a serum or gamma-globulin dilution in the same buffer. The mixtures were then kept at 44° C for 30 minutes. The test was then performed by means of the egg test.

7. Desoxyribonuclease (DNase) and Ribonuclease (RNase)

Here we were working with crystalized preparations supplied by the Worthington Co., Freehold, N. J.

8. Determination of NA Concentration

The determination of the entire NA contents (RNA and DNA) of the fractions was performed by means of the UV spectrophotometer in accordance with the method described in the first communication (/1/).

9. Ultracentrifugation

The experiments were performed in a preparative ultracentrifuge supplied by the firm of Beckman Instruments, Ltd., Spinco Model L. The NA fractions, respectively, the virus preparations, were dissolved for this purpose in 0.05-m. phosphate buffer with a p_H of 7.0.

Results

1. The Infectiousness of NA Fractions

All NA fractions obtained from chicken embryos infected with the EEE virus had a demonstrable infectiousness for a chicken egg that had been incubated for 10-11 days and that had been fertilized. The embryos, on the average, died with 24-48 hours after the infection, just like after an infection with EEE virus. The infection titer of the NA fractions here averaged around 4 powers of 10 below those of the virus preparations which had been made from the same initial material and which had been introduced in the same volume (see Table 1).

Nr. (a)	Viruspräparat [LD_{50}/ml] (b)	NS-Fraktion [LD_{50}/ml] (c)
1	8,1	2,9
2	7,3	3,3
3*	6,5	3,1
4*	7,7	3,7
5*	6,3	3,2
Durchschnitt: (d)	7,2 ± 0,9	3,3 ± 0,4

Table 1. Infectiousness of Virus Preparations and NA Fractions for a Fertilized Chicken Egg Incubated 10-11 Days. (*) A NA fractions have been purified by means of alcohol precipitation. Legend: a--number; b--virus preparation; c--NA fraction; d--average.

In tissue cultures of embryonal chicken cells it was also possible to establish the infectiousness of the NA fractions with the help of the plaque test. Their infection titer however was always smaller when this system was used than when the egg test was used. The virus itself behaved in the exact opposite fashion: Its titer was about 2 powers by 10 higher in the plaque test than in the egg test. The difference of infectiousness between the NA fractions and corresponding virus preparations therefore increased in the plaque test to about 7 powers of 10 or more (see Table 2).

The speed of plaque development as well as the size of the plaques were almost identical for virus fractions and NA fractions.

	a	b	a	b	a	b	PBE/ml**
(a) Verdünnung	10^{-6}		10^{-7}		10^{-8}		
Virus	(c) nicht aus-zähl-bar		127	148	13	12	$2,75 \cdot 10^{9}$
(b) NS-Fraktion	10^{0}		10^{-1}		10^{-2}		
1	20	6	5	2	3	0	$7 \cdot 10^{1}$
2	4	3	4	3	0	0	$7 \cdot 10^{1}$
3	16	4	47	32	3	6	$7,9 \cdot 10^{2}$
4	2	0	9	1	1	0	$1 \cdot 10^{0}$

Table 2. Plaque Count in a Virus Preparation and NA Fractions as a Function of the Dilution. (**) The PBE/ml for the NA fractions were determined on the basis of the plaque count in dilutions of 10^{-1}. Legend: a--dilution; b--NA fraction; c--could not be counted; PBE/ml--plaque-forming units per milliliter.

Earlier (/10/) a phenomenon was described to the effect that there is no linear proportion between the plaque count and the dilution when we use NA fractions in the plaque test; this same phenomenon was also observed once again in our experiments here. In virus preparations, such a linear relationship however is certainly present (see Table 2). An accurate titer in PBE/ml can therefore be given only for virus preparations and it cannot be given for NA fractions. Of course, it seems as if there might be an approximately linear proportion in the case of the latter between the dilution and the plaque count when we work with dilutions of 1:10 and 1:100 and it seems as though the plaque count would be irregular only in the case of the undiluted NA fractions. This is why all infection titers of NA fractions, which we have in this study and which are given in terms of PBE/ml, refer to the number of plaques determined in a dilution of 1:10 of the particular preparation.

The previously described irregularity of the dose-effect relationship could be observed also when we tested the infectiousness of raw NA fractions in the incubated and fertilized chicken egg (see Figure 1). Here, the success of the infection was likewise greater with a lower concentration than with a higher concentration.

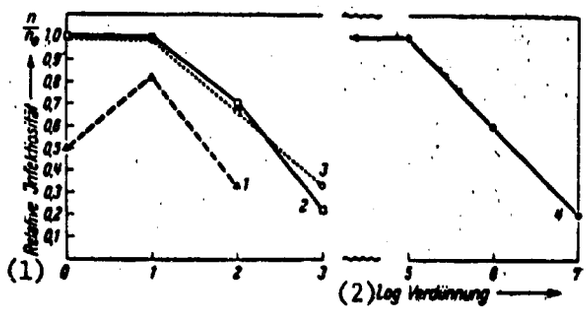

Figure 1. Dose-Effect Curves. Curve 1: Raw NA fraction. Curves 2 and 3: Alcohol-precipitated NA fractions. Curve 4: Intact EEE virus. n_0 = number of embryos inoculated. n = number of EEE-infected embryos. Legend: 1--relative infectiousness; 2--log dilution.

There is reason to suspect that this irregularity is due to the admixtures which are contained in the preparations in addition to the infectious principle and which, in higher concentrations, interfere with the onset of the infection.

2. The Inhibitorily-Acting Substances in the NA Fractions

According to the investigations on the physical and chemical properties of the NA fractions (/1/), these fractions, in addition to the infectious RNA molecules with a molar weight of about $2 \cdot 10^6$, contain various other admixtures:

(a) Small-molecular ribonucleotides,

(b) High-molecular DNA,

(c) High-molecular polysaccharides,

(d) Small-molecular peptides or aminoacids.

The small-molecular admixtures a and d both can be removed by precipitating the high-molecular compounds by means of alcohol and by thus separating them.

For this purpose, we mixed 1 volume of NA fraction with 2 volumes of pure, cooled /refrigerated/ ethanol. After centrifugation of most of the immediately developing precipitates (5 minutes at 3,000 rpm), these /precipitates/ were washed twice with a mixture of 0.02-m. phosphate buffer, pH 7.0 and ethanol in a ratio of 1:2. The washed precipitates were then dissolved in the original volume of 0.02-m. phosphate buffer.

As we can see in Table 3, the infectiousness of the NA fractions is increased as a result of this purification method. The dose-effect curves now look like the curves obtained with the impact virus (see Figure 1, curves 2 and 3).

Nr. (a)	Präparat (b)	Gesamt-NS [mg/ml] (c)	Infektiosität [LD_{50}/ml] (d)	Spezifische Infektiosität [LD_{50}E**/mg NS] (e)	Faktor (f)
1	roh (g)	1,07	1,7	47	4,9
	(h) präzipitiert	0,66	2,2	231	
2	roh (g)	1,31	2,9	832	4,25
	(h) präzipitiert	0,96	3,6	3550	

Table 3. Increase in Specific Infectiousness of NA Fractions by Means of Alcohol Precipitation. (**) LD_{50} units. Legend: a--number; b--preparation; c--total NA; d--infectiousness; e--specific infectiousness; f--factor; g--raw, h--precipitated.

These experiments told us that the low-molecular ribonucleotides or the low-molecular peptides, respectively, aminoacids, do exercise an inhibitory effect upon the infectiousness of the virus-RNA.

As far as the impurities of NA fractions, which cannot be removed by means of precipitation with alcohol, are concerned, it seems that the high-molecular DNA likewise exercises an inhibitory activity because the treatment of an NA fraction with 1 gamma DNase/ml for 15 minutes at room temperature likewise increased the infectiousness. The irregular course of the dose-effect curves in this case remained practically unchanged (see Table 4).

Verdünnung NS-Fraktion (a)	Enzym-Konzentration [γ/ml] (b)	(c) Infektionserfolg RNase	DNase	Kontrolle (d)
1:5	1,0	0	7/10**	7/10
	0,1	0	8/10	
	0,01	0	9/10	
1:10	1,0	0	10/10	9/10
	0,1	0	10/10	
	0,01	0	9/10	
1:100	1,0	0	9/10	5/10
	0,1	0	8/10	
	0,01	0	9/10	
1:1000	1,0	0	4/10	3/10
	0,1	0	1/10	
	0,01	0	4/10	

Table 4. Success of Infection of an Alcohol-Precipitated NA Fraction as a Function of the Dilution Before and After Incubation with DNase. (**) Numerator: number of dead embryos; denominator: number of inoculated embryos. Legend: a--dilution, NA fraction; b--enzyme concentration; c--results of infection; d--control.

The figures for the infectiousness of NA fractions in terms of LD_{50}/ml, computed according to the method of Reed and Muench (/11/), can be correct only if the dose-effect curves are regular. As we can see from the experiments described, this applies quite extensively to the alcohol-precipitated NA fractions. In the raw NA fractions, however, this kind of evaluation is not permissible and can give us only approximate values.

3. Behavior of NA Fractions Toward Serums

With the help of a gamma-globulin fraction of an antiserum against tabacco mosaic virus (TMV), Gierer and Schramm (/2/) were able to show that intact TMV was still neutralized by gamma-globulin dilutions which did not influence the infectiousness of a corresponding RNA. From this the authors were able to conclude that the infectious RNA molecules no longer have any antigenic proteins which are essential for their infectiousness.

Unfortunately, the results, which we were able to obtain in such experiments with our NA fractions, were by no means as conclusive.

The difficulty rise in the apparently extremely high lability of the infectious RNA with respect to the serums as such.

As we can see in Table 5, NA fractions are inactivated by standard serums or by standard-gamma-globulin fractions. Here there is a definite connection between the protein concentration and the inactivation effect.

The infectiousness of the virus preparations on the other hand is not influenced by the standard serum -- as was to be expected. We were unable to dilute the antiserums available to us sufficiently thoroughly to achieve a figure below the protein concentration that is critical for the NA fractions, when we had an intact virus whose neutralization could still be established; this is why we did not continue studies of this kind any further, for the time being.

Serum	Verdünnung (a)	Virus-präparat $[LD_{50}/ml]$ (b)	NS-Fraktion $[LD_{50}/ml]$ (c)	Serum-protein-Konzentration [γ/ml] (d)
(e) Normales Pferde-Serum	1:50	6,1	0,6	1230
	1:100	6,2	0,0	615
	1:200	6,4	1,3	307
	1:400	6,0	1,2	153
(f) Normales Kaninchen-Serum (γ-Globulin-Fraktion)	1:16	7,0	0	584
	1:128	6,9	1,1	73

Table 5. Influence of Standard Serum and Standard Gamma-Globulin upon the infectiousness of Virus Preparations and NA Fractions. Legend: a--dilution; b--virus preparation; c--NA fraction; d--serum-protein concentration; e--standard horse serum; f--standard rabbit serum (gamma-globulin fraction).

With the help of the experiments with the standard serums we were not yet able to decide whether the nonspecific inactivation of NA fractions is based on a protein effect as such or on the presence of a serum-RNase, as was assumed by Alexander and his associates (/3/). We were able to observe a complete inactivation of an NA fraction also after 1/2-hour incubation with 20 gamma DNase/ml at room temperature although -- as we described earlier-- DNase, in concentrations of 1 gamma/ml or less is capable of increasing the infectiousness. In this case, likewise, we still do not know whether the 20 gamma DNase-protein/ml or a contamination of the enzyme with RNase are responsible for this inactivation.

4. Material Produced after Infection with NA Fractions

Chicken embryos, which had been infected with NA fractions and which had died of this infection, again contained an infectious principle. We now had to answer the question as to whether this /principle/ differs from the standard EEE virus and whether, for instance, it has properties which correspond to those of the infectious RNA in the NA fractions.

The infectiousness of virus preparations and NA fractions is influenced in various ways as a result of varying treatment -- very often even in the opposite sense.

These differences in behavior are indicated in Table 6.

Nr. (a)	Material	(b) Viruspräparate [LD_{50}/ml] log	[PBE/ml] log	NS-Fraktionen (c) [LD_{50}/ml] log
1	Ausgang (d)	8,1	9,5	3,0
	alkoholpräzipitiert (e)	0	1,3	3,5
	25 γ RNase 30 Min. bei 37°C	7,7	9,1	0*
	25 γ RNase 4 Stdn. bei 37°C	6,7	9,0	0
2***	Ausgang (d)			2,9
	0,01 γ RNase 15 Min. bei 10°C			0
	0,01 γ DNase 15 Min. bei 10°C			3,5
3***	Ausgang (d)	7,4		
	20 γ RNase 15 Min. bei 20°C	7,7		
	alkoholpräzipitiert (e)	0		
	Ausgang (d)	3,7**		3,8
	55 Min. 40000 U/Min.	1,0		3,6
	25 γ RNase 20 Min. bei 20°C	3,6		0
	25 γ DNase 20 Min. bei 20°C	3,8		0
4***	(f) Normalserum 1:100	6,2		0
	EEE-Antiserum 1:100	6,4		2,0

Table 6. The Differences Between Virus Preparations and NA Fractions Under Different Treatment Methods. (*) 0 = infectiousness cannot be established or demonstrated. (**) The virus is diluted in NA fractions from standard chicken embryos. (***) NA fractions precipitated with alcohol. Legend: a--number; b--virus preparations; c--NA fractions; d--initial; e--precipitated with alcohol; f--standard serum; bei--at; Stdn.--hours; U/Min.--rpm.

Precipitation with alcohol, even in undiluted virus preparations, completely destroys the infectiousness; on the other hand -- as we said before -- irregularly increases the infectiousness of NA fractions. Relatively high concentrations of RNase (20-25 gamma/ml) on the other hand do not have any negative effect upon undiluted or diluted virus preparations, whereas NA fractions with comparable initial infectiousness are completely inactivated already by a 2,000-times smaller RNase concentration (see Table 6, No. 2). High-rate centrifugation in the ultracentrifuge reduces the infectiousness of virus preparations always by an amount that is much, much greater than that of the NA fractions. This cannot be blamed on the differing viscosity of the solution. In one experiment (Table 6, No. 2), a virus preparation was so diluted in an NA fraction, made from normal, non-infected chicken embryos, that its initial infectiousness was the same as that of an infectious

NA fraction. The entire NA concentration was also adjusted to the same identical values and both preparations were then centrifuged in a parallel fashion for 55 minutes at 40,000 rpm. The infectiousness of the virus preparation as a result dropped by 99% whereas that of the NA fraction dropped by only about 40%.

In experiments concerned with the identification of the infectious principle, formed as a result of infection with NA fractions in chicken embryos, we confined ourselves to examining its behavior toward RNase and alcohol-precipitation as well as specific antiserum.

For each mixture we homogenized a chicken embryo, which had died as a result of infection with NA fraction, in the frozen state, with 4 milliliters of 0.1-m. phosphate buffer, p_H 7.5. The cell rubble was centrifuged as usual. The supernates involved here were divided into two halves, each one of which served as control in each case.

As we can see from Table 7, the infectious principle in such chicken embryos was not RNase-sensitive but was completely inactivated by treatment with alcohol. The infectiousness of the controls corresponded to that of the virus preparations which can be obtained in the same manner from virus-infected chicken embryos. The incubation of various virus dilutions with an equal concentration of EEE antiserum, in each case, overnight at +4° C, reduces the infectiousness down to 1% of the control which had been incubated in the same manner with standard serum.

The infectious principle from chicken embryos, which had been infected with NA fractions, accordingly behaved just like intact EEE virus in the directions and along the lines mentioned.

Nr. (a)	Behandlung (b)	[LD_{50}/ml]
1	Normalserum 1:4 = Kontrolle EEE-Antiserum 1:4 (c)	7,4 5,3
2	Puffer 15 Min. 20°C = Kontrolle 25 γ RNase 15 Min. 20°C (d)	7,4 8,4
3	Kontrolle (e) Alkohol-Präzipitation (f)	8,0 0

Table 7. Behavior of Infectious Material Formed After Infection with NA Fraction in the Chicken Embryo. Legend: a--number; b--treatment; c--standard serum 1:4 = control; d--buffer 15 minutes 20° C = control; e--control; f--alcohol precipitation.

5. Initial Material for Infectious RNA

In an earlier work we reported that we were able to obtain an infectious

NA fraction from EEE-infected mouse brains by means of phenol only when phenol was already present during the homogenization of the frozen brains (/10/).

In contrast to this we have the experiences of Colter and associates (/5, 6/), who ground the infected cells or tissues in the frozen state first of all in a mortar, who then homogenized with buffer, centrifuged the cell rubble, and were afterward able to isolate the infectious RNA preparations from the supernates by treatment with phenol.

Our observation, which we had made during the extraction of EEE-infected mouse brains, could now be confirmed quite extensively when we used infected chicken embryos:

Four frozen, EEE-infected chicken embryos were homogenized with 12 milliliters of 0.02-m. phosphate buffer, p_H 7.0, for 5 minutes under refrigeration. Of the homogenate, 2 milliliters were centrifuged for 10 minutes at 3,000 rpm and at a temperature of + 4° C. The supernate is then called a virus preparation. The remaining homogenate was centrifuged for 3 minutes at 13,000 rpm, at +4° C; the supernate was mixed with 20 milliliters of 80% phenol, it was heavily agitated /shaken/ for 8 minutes under cooling /refrigeration/, and it was then again centrifuged. The water phase was removed and we then agitated twice more in the same fashion with phenol. The further processing then was accomplished in the usual manner, including the alcohol precipitation (see material and methods). In all experiments, the material, precipitated by alcohol, was dissolved in 0.02-m. phosphate buffer, p_H 7.0. The volume corresponded to that of the initial material.

The virus preparation contained 7.25 LD_{50}/ml. Its infectiousness thus was on the average level determined in the other experiments. The NA fraction, on the other hand, only had 2.02 LD_{50}/ml at a total NA concentration of 940 gamma/ml or a specific infectiousness of 2.05 (log) LD_{50}/mg NA = 112 LD_{50} - units/mg NA ($LD_{50}E$/mg NA).

The average infectiousness for alcohol-precipitated NA fractions, in the usual extraction, however is 3.2-3.3 LD_{50}/ml, with an average overall concentration of NA amounting to 1.6 mg/ml. This corresponds to a specific infectiousness of 990 $LD_{50}E$/mg NA /E--units/. The preparation which was homogenized without phenol thus only had about 7% of the customary average infectiousness, at a total NA concentration which at any rate amounted to 60% of the otherwise average concentration. Its specific infectiousness was thus reduced down to 11% of the otherwise average value.

In order to explain these findings further, we made the following experiment:

The supernates of homogenates, which were made as described before, were incubated with 10 gamma RNase/ml, for 15 minutes at room temperature, prior to treatment with phenol. The raw and alcohol-precipitated NA fraction obtained from this was then checked for infectiousness in the egg test.

In neither of the two preparations were we able to establish any infectiousness, although the entire NA concentration of the alcohol-precipitated fraction amounted to 47% of the otherwise customary average value.

These findings show us that the extractable initial material is available for the infectious NA fractions in an RNase-sensitive form. The findings of Colter and associates (/6/) and of Franklin and associates (/4/) conflict with this. In these works, which involved Mengo-virus, respectively, mouse encephalomyelitis virus, the authors were able to show that treatment of the homogenate-supernates with RNase, prior to phenol treatment, does not influence the infectiousness of the final products.

In other words, in the case of the EEE-infected cells, it was possible to establish the RNase-sensitivity of the initial material; this necessarily means that this initial material could not possibly be the EEE-virus elementary particles because these particles resist RNase. At the same time one might suspect then that infectious RNA cannot be extracted from the elementary virus particles of American horse encephalomyelitis with the method described here. This was proved quite clearly in a purified virus preparation of the West type of American horse encephalomyelitis (WEE). The preparation, which contained $2.7 \cdot 10^{10}$ PBE/ml, was treated with phenol 3 times. The final product did not contain any demonstrable NA and did not have any infectiousness either. From WEE-infected cells, on the other hand, we can obtain infectious NA fractions by means of the same method, just as in the case of EEE.

Discussion

If we homogenize chicken embryos, which had died of infection with EEE virus, in the presence of 80% phenol, and if we extract the proteins in three further cycles with phenol, then the resulting NA fractions are infectious for chicken eggs that have been incubated for 10-11 days. There they stimulate the multiplication of normal EEE virus -- to the extent that this was examined here. Plaques can also be produced with such NA fractions on tissue cultures of embryonal chicken cells. The difference in the infection titer between the NA fraction and the corresponding virus preparation, which is as much as 10^4 in the egg test, goes up to 10^7 - 10^8 in the plaque test.

In most of the studies published on infectious virus-RNA preparations in the past (/2-6/), we find a difference of 2-3 powers of 10 between the infectiousness of RNA and virus preparations. In these cases, however, it was possible to demonstrate with a great degree of probability that the infectious RNA was extracted from the infectious elementary virus particles themselves. In the infectious NA fractions from chicken embryos infected with EEE virus, this is not the case. If we homogenize the infected embryos without the presence of phenol and if we treat the virus-containing homogenate-supernates only after centrifugation of the cell rubble with phenol, then the specific infectiousness (LD_{50}-units per mg total NA) of the final products will drop to about 10^{-4} of the otherwise average level. Colter and associates (/5, 6/) however use this method quite fundamentally here. They were able to

incubate the homogenate-supernates with RNase, even prior to treatment with phenol, without reducing the infectiousness of their RNA preparations as a result (5). This finding has in the meantime been confirmed by Franklin and associates (4) in connection with the production of infectious RNA fractions from tissue infected with mouse encephalomyelitis virus. But if we use this technique in the case of chicken embryos infected with EEE virus, then the NA fractions obtained will not have any demonstrable infectiousness.

The initial material for infectious RNA in these NA fractions is thus sensitive to RNase. The presence of phenol already during the homogenization of the tissues obviously causes the cell's own RNases, which are activated during homogenization, to be rapidly inactivated again and the extractable virus material, which is sensitive to RNase, is not destroyed.

The elementary EEE virus particles themselves resist the enzyme. In the case of the West type of American horse encephalomyelitis (WEE) we were finally able to prove that no demonstrable RNA is extracted from purified virus concentrates ($2.7 \cdot 10^{10}$ PBE/ml) according to the phenol method of Gierer and Schramm (2) and that such preparations do not have any infectiousness at all. In the extraction of WEE-infected tissues, however, we get an infectious NA fraction, as in the case of EEE.

A short time ago, we were able to produce infectious RNA from the elementary virus particles of WEE through extraction with phenol at high temperatures (/14/ E. Wecker, Virology 7, 241, 1959).

The findings described can only be interpreted in the following manner: In the case of tissues infected with EEE and WEE, it is not the elementary virus particles themselves which represent the initial material of the infectious RNA; instead, the RNA is here extracted from another virus-specific material which already contains it /RNA/ in a biologically active form. A comparison of the infectiousness of NA fractions and of corresponding virus preparations thus is not readily possible in this case. In addition we have the fact that the NA fractions, obtained according to the method described here, also contain smaller polyribonucleotides, DNA, high-molecular polysaccharides, and aminoacids or smaller peptides, in addition to the actual infectious high-molecular RNA with a probable molar weight of $2 \cdot 10^6$ (1).

Some of these accompanying substances seem to have an inhibitory effect. This emerges from the fact that the infectiousness of raw NA fractions is increased by alcohol precipitation.

As we showed in the first communication (1), the small polyribonucleotides as well as the aminoacids or small peptides can be removed by means of this purification step.

A further infectiousness increase then results from a careful treatment of the preparations with DNase (1 gamma DNase/ml or less). Larger DNase concentrations (20 gamma/ml) on the other hand completely destroy the infectiousness. Whether this is based on a pure protein effect or on a possible

contamination of the DNase preparation with RNase cannot be determined right now.

Likewise we still do not know why standard serums have a strong inhibitory effect upon the infectiousness of the NA fractions. Here again, according to the work of Alexander and his associates (3), we must expect that the presence of RNase in the serums is the reason for this. But it will have to be determined whether this applies also to the gamma-globulin fraction of serums.

Working with infectious NA fractions, which can be obtained from virus-infected tissues according to the technique described here, entails additional difficulties because of their complex composition; nevertheless it seems worth while to continue such studies. In this way it is possible to get infectious virus-RNA also when the extraction of the RNA from the elementary virus particles themselves cannot be accomplished with the customary methods. Moreover, it would appear to be worth while to investigate the occurrence of infectious virus-RNA, outside the elementary virus particles, qualitatively and quantitatively in the future.

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