Investigations on a Tissue Antagonist of Interferon (TAI)[1] (34343)

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In the course of investigations on viral antagonist(s) of interferon, a chance observation led to the demonstration of a tissue antagonist of interferon activity (TAI). This antagonist is present in extracts of the human chorionic or amniotic membranes. We report herein the results of experiments on the demonstration, the purification, and some of the biophysical properties of TAI. The summary of these data has been presented in a preliminary report (1).

I. Material and Methods. 1. Tissue culture and media. For cell growth Eagle's medium (MEM 0111), supplemented with tryptose phosphate broth 2.95 g/liter and 10% heat-inactivated calf serum was employed. For the maintenance of the cells the serum concentration in the medium was decreased to 2%.

Amniotic and chorionic membranes were obtained from normal deliveries. The membranes were immediately separated from the placenta, washed four times in 150 ml of saline and placed in MEM 0111 medium (5% calf serum) for 24–48 hr. The medium was changed daily thereafter.

For cell cultivation the amniotic membranes were trypsinized, and the cells were suspended in growth medium at a concentration of 400,000 cells/ml/tube. The tubes were incubated vertically in an airtight box saturated with air and 5% CO_2 at 37°.

2. Extraction of the tissue culture antagonist from the human amniotic or chorionic membrane. The amniotic or chorionic membranes were obtained and washed as described previously. However they were frozen at —80° after the last washing. The tissues were then completely dried by lyophilization in a vacuum at —40°. Lyophilized membranes were pulverized in a Grinder (Beckman Grindex). The fine powder was then rehydrated in Eagle's medium at a concentration of 2 g of dry weight/100 ml and agitated with a magnetic stirrer for 18 hr at 4°. The extract was clarified by low speed centrifugation (5000 rpm for 30 min) and then by high speed centrifugation (90,000*g* for 4 hr) in a preparative Spinco ultracentrifuge.

The supernatant was dialyzed at 4° against 0.125% tricholoroacetic acid for 18–20 hr. The precipitate was eliminated by centrifugation at 5000 rpm for 20 min and the supernatant was redialyzed against Eagle's medium. The residual protein concentration in the preparation used as TAI, was $\leq$62.5 mg/100 ml.

3. Viruses. (a) The Indiana strain of *vesicular stomatitis virus* (VSV) was serially propagated in L cells. Titrations were performed by routine plaque methods with agarose and 2 percent calf serum.

(b) *Newcastle disease virus* (Hertfordshire strain) was obtained through the courtesy of Dr. Béla Lomniczi. The virus was grown in the allantoic cavity of 9-day-old embryonated Leghorn eggs. The stock suspension was titered in chick embryo fibroblasts by plaque methods described above.

4. Interferon preparations. (a) Human interferon was obtained in human leukocytes as described by Gresser (2) and modified by Falcoff *et al.* (3). Briefly, leukocytes were separated from plasma after sedimentation with dextran. The white cell suspension was separated from the plasma by centrifugation and washed. The cells were then infected with NDV at m.o.i. = 100 and incubated for 3 hr at 37°. The unadsorbed virus was eliminated by low speed centrifugation (5000 rpm for 30 min). The cells in the sediment were

[1] This study was supported by Grant No. 66 00 265 Délégation Générale á la Recherche Scientifique, Paris.

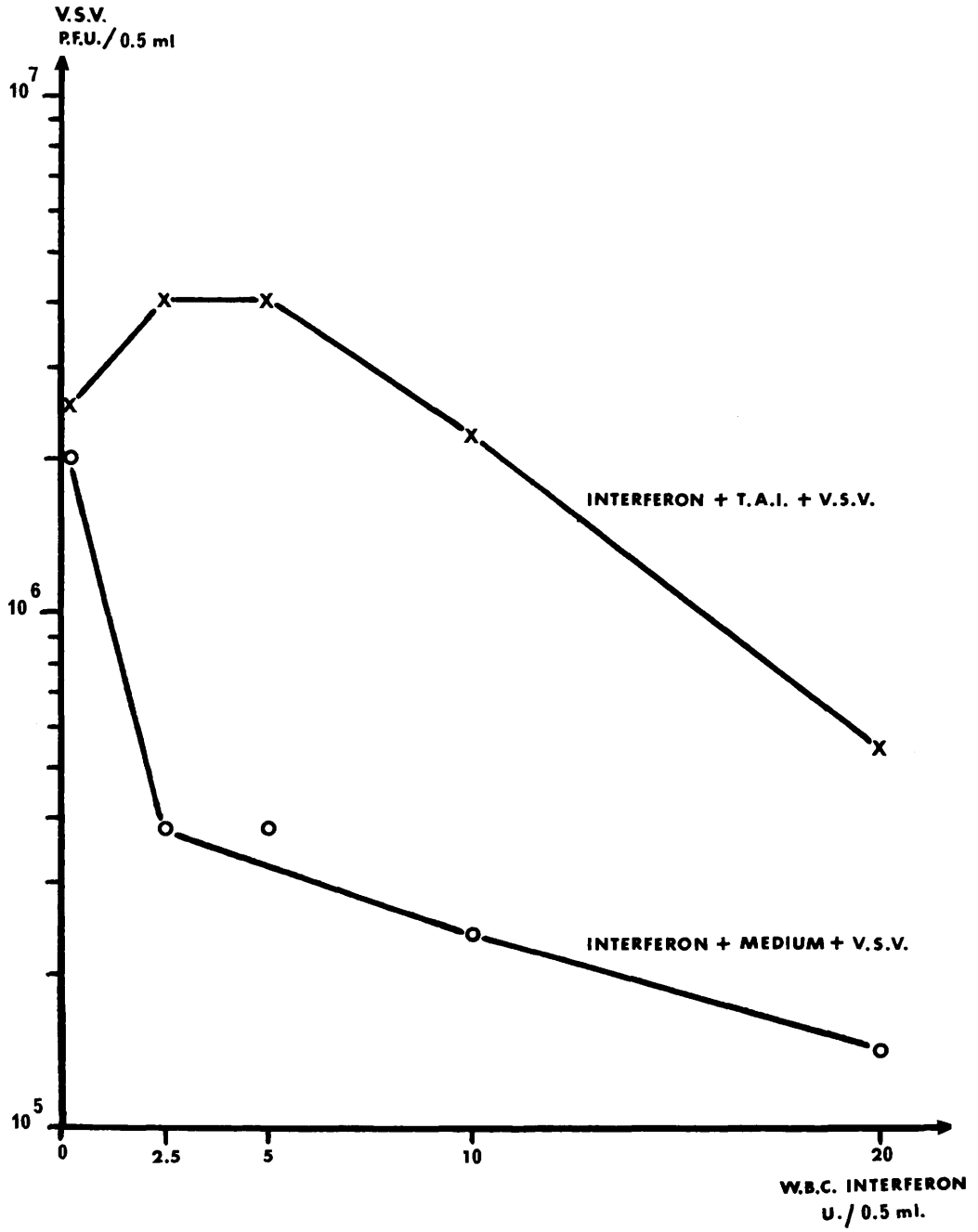

FIG. 1. Action of TAI on WBC Interferon; human amniotic cells were treated with increasing concentrations of interferon for 4 hr. The cells were washed and challenged with TAI at a concentration of 2 g/100 ml. A significant decrease of anti-interferon effect was observed.

resuspended in MEM 0111 medium plus 2% human serum and incubated for 24 hr at 37°. At the end of the incubation period, the cells were eliminated by low speed centrifugation. The residual infectious virus in the supernatant was eliminated by lowering the pH to 2 with *N* HCl for 5 days at 4°. The pH was then readjusted to 7 with *N* NaOH. This

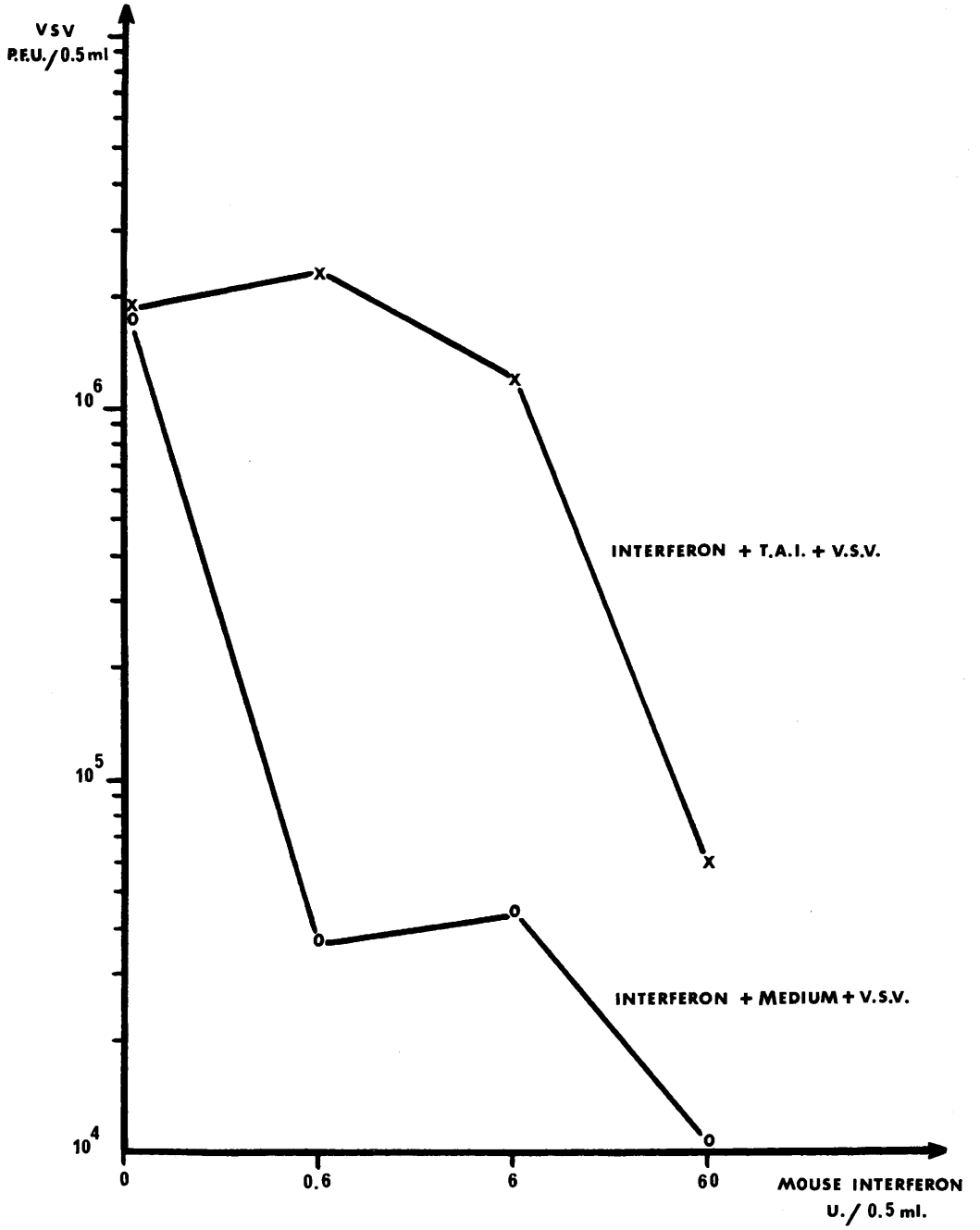

FIG. 2. Action of TAI on mouse interferon; L cells were treated with increasing concentrations of interferon for 4 hrs. The cells were washed and challenged with TAI at a concentration of 2 g/100 ml. A significant decrease of anti-interferon effect was observed.

suspension represents the crude interferon preparation employed in the experiments. Further purification was obtained by chromatography in Sephadex G 100 as described previously (3).

(b) *Mouse interferon* was obtained by infecting Balb/c cells (MSVI strain) with NDV at the multiplicity of infection = 200 (4). After 24 hr of incubation the pH of the tissue culture fluid was adjusted to 2 with *N*

HCl and kept at 4° for 5 days. Purification was obtained in the Sephadex G 100 column as previously described (3).

(*c*) *Titration of interferon. 1. By CPE inhibition.* Interferon was incubated after serial twofold dilutions with L cells grown in vertical hemolysis tubes. After 4 hr of incubation the cells were washed and infected with VSV, m.o.i. = 1. The titer of interferon preparation was evaluated by the dilution which inhibited the cytopathic effect (CPE) in approximately 50% of the cell population.

2. *By viral yield inhibition.* Serial twofold dilutions of interferon were incubated either with primary amniotic cells or with L cells. The cells were incubated for 4 hr, washed and further incubated for 18 hr. They were then infected with VSV, m.o.i. = 10, incubated at 37° for 16 hr. The viral yield was determined by plaque titration and compared to suitable control cultures.

(d) *Treatment of TAI with proteases.* The TAI was diluted 1:2 with a solution containing 250 μg/ml of twice crystallized trypsin (Choay). The effect of trypsin was blocked with a trypsin inhibitor (Iniprol Choay, 2500 U/ml). Pepsin was employed 250 μg/ml at 1:2 dilutions for 1 hr at 37°. The solution was adjusted to pH 2, then incubated at 37° for 1 hr. At the end of the incubation period the pH was readjusted to 7.

II. Results. Demonstration of the anti-interferon activity of tissue antagonist. Human amniotic cell cultures were treated with increasing concentrations of crude or purified human interferon. After 4 hr, the cells were washed and incubated with the TAI at a concentration of 2%. In control preparations the TAI was replaced by medium. The cells were further incubated for 18 hr before addition of VSV, m.o.i. = 10. After 18 hr, VSV CPE was recorded and the cultures were frozen at −80° for titration in L cells.

In cells treated with 2.5 units of human interferon, the yield of VSV was decreased 10-fold. With increasing concentrations of interferon only a slight additional reduction in viral yield was obtained. In cells treated with TAI, the protective effect of interferon was completely abolished up to the concentration of 5 units of interferon/0.5 ml. With further concentration, the inhibitory effect of interferon reappeared, however no parallelism between the 2 curves was observed. The antagonist was still effective in reversing the protective effect of 20 units of interferon (Fig.).

Species specificity of interferon antagonist. The antagonist proved to be active in L cells reversing the effect of 60 units of unpurified mouse interferon (Fig. 2). The dose–response curve obtained in this system was comparable to the one obtained in human cells. Thus, the antagonist was shown to cross the species barrier. Because of the simplicity of the mouse interferon system, all further experiments were undertaken utilizing purified mouse interferon in L cells.

The dose–response relationship between a constant amount of interferon (200 units) and an increasing concentration of the antagonist is summarized in Fig. 3. The results show that the antagonist has no demonstrable direct effect on VSV replication. In spite of the potency of interferon which decreases VSV multiplication almost 1000-fold, the antagonist was effective against the established antiviral state. There was a linear correlation between the concentration of the extract and its anti-interferon activity between 0.25 and 2 g/100 ml. At the concentration of 2 g/100 ml the activity of interferon was decreased approximately 100-fold.

Effect of antagonist on the production of interferon induced by NDV in L cells. Since TAI decreased the action of interferon even when the antiviral state was already established (*i.e.* 4 hr after addition of interferon) experiments were undertaken to determine whether TAI affected the production of interferon induced by viruses.

The L cells were grown in 500-ml Roux flasks. When the cell sheet was confluent, the growth medium was removed and the cells treated for 18 hr at 37° with 50 ml of the antagonist suspended in tissue culture medium at a concentration of 2 g/100 ml. At the end of the incubation period, the cells were washed and challenged with NDV, m.o.i. = 200.

The supernatants were collected at 24, 48, and 72 hr after the challenge with NDV. Each time after the removal of the superna-

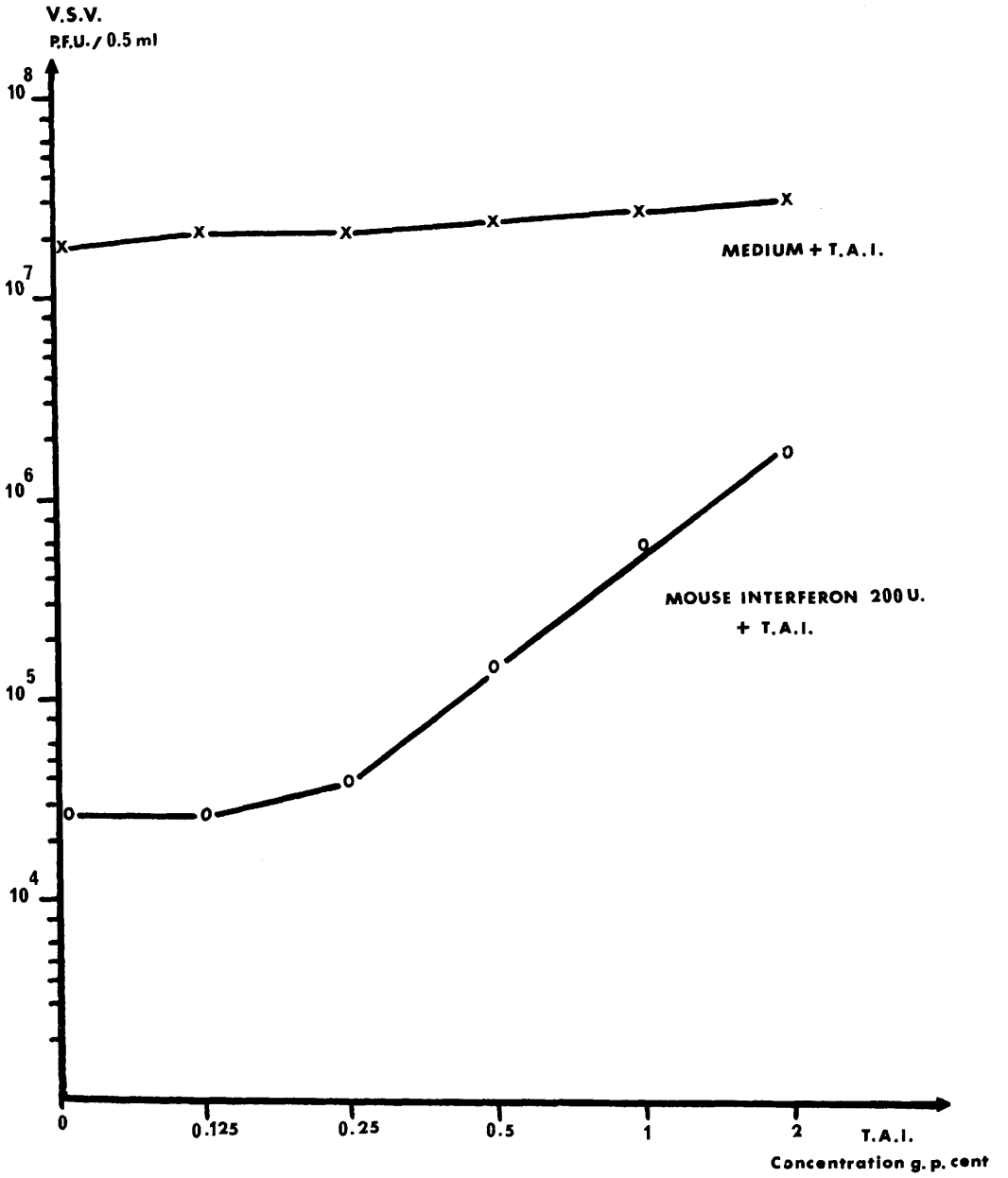

FIG. 3. Dose response relationship between tissue extract and VSV in the presence of 200 U of interferon; L cells were treated with 200 U of mouse interferon for 4 hr. The cells were washed and new medium containing increasing concentrations of TAI was added for 18 hr. After removal of the medium, the cells were challenged with VSV and the viral yield was measured after the first cycle of multiplication.

tant, fresh medium was added to the cells. Interferon was separated from the virus and tested as described in "Material and Methods." Results summarized in Table I show that TAI has no significant effect on the production of interferon.

Direct inter-action TAI–interferon. The previous experiments suggested that TAI act-

TABLE I. Action of TAI on the Production of Interferon.

L cells (hr):	Interferon titer/ml in supernatant of L cells after: 24	48	72
TAI (18 hr) + NDV	256[a]	2048	32
Medium (18 hr) + NDV	256	2048	32

[a] Reciprocal of the dilution which inhibits 50% of the CPE induced by VSV.

ed intracellularly. It seemed however important to exclude the possibility of a direct action of TAI on interferon. Accordingly 200 units of interferon were mixed with an equal volume of TAI. In control preparations TAI was replaced by an equal amount of medium.

The preparations were incubated for 2 hr at 37° and then added to L cells. The mixtures were removed after an incubation period of 4 hr. The cells were then challenged with VSV. As shown in Table II no significant change in action of interferon could be observed. Consequently the antagonist neither prevented the fixation of interferon on the cells nor the establishment of the antiviral state.

Some physico-chemical properties of TAI. (a) *Partial purification of TAI by precipitation of the macromolecular fraction.* The crude TAI preparation was mixed with trichloroacetic acid (TCA) 5% solution for 24 hr at 4°. The precipitate was removed by centrifugation at 5000 rpm for 30 min. The pH was readjusted to 7 by dialyzing the preparation with several changes of Eagle's medium. All anti-interferon activity was recovered in the acido-soluble fraction. This purified TAI was employed in subsequent experiments.

TABLE II. Interaction Interferon–TAI.[a]

No.	Incubation at 37° for 2 hr of:	VSV titer (PFU/0.5 ml) on L cells
	Interferon 200 U.	
1	+ TAI	6×10^{3}[b]
2	+ Medium	6×10^{3}
	Interferon 4 hr	
3	+ TAI 18 hr	5.7×10^{4}
4	+ Medium	6×10^{8}
5	Medium + TAI 18 hr	5.8×10^{4}
6	VSV challenge virus	6.2×10^{4}

[a] Interferon and TAI were mixed 1:2 and incubated at 37° for 2 hr. The inhibitory effect of interferon was unchanged (1–2–6). The same TAI preparation when added 4 hrs after interferon decreased ≃ tenfold the action of interferon (3–4–5–6).

[b] Yield of VSV after the first cycle of multiplication.

(b) *Effect of proteases on TAI.* When TAI was treated with trypsin or with pepsin as described in "Material and Methods," the biological activity of TAI was either not impaired or even increased. A similar effect was observed when TAI was treated first with pepsin and subsequently with trypsin. Adequate controls showed that: (a) neither of these enzymes had any effect on VSV replication, and (b) both destroyed the biological activity of 200 interferon units (Table III).

TABLE III. Action of Proteases on TAI.

	VSV titer (PFU/0.5 ml) on L cells: Series with 200 U interferon	Series without interferon
TAI	4×10^{5}[a]	2×10^{7}
TAI + trypsin (500 μg/ml) + iniprol	2.7×10^{5}	10^{7}
+ Medium + iniprol (5000 U./ml)	1.4×10^{6}	2×10^{7}
+ Pepsin (500 μg/ml) (pH 2)	1.5×10^{6}	10^{7}
+ Medium (pH 2)	2×10^{5}	10^{7}
Interferon, 200 U + Trypsin (500 μg/ml) + iniprol	8×10^{6}	–
+ Pepsin (500 μg/ml) (pH 2)	1.3×10^{7}	–
Control interferon, 200 U	1.5×10^{4}	–
challenge virus VSV	–	10^{7}

[a] Yield of VSV after the first cycle of multiplication.

(c) *Effect of heat on TAI.* The TAI preparations were heated for 1 hr at 56, 75, and

95°. As shown in Table IV, a significant destruction of TAI was only observed when the preparation was heated at 95° (Table IV).

TABLE IV. Effect of Heat on TAI.

	VSV titer (PFU/0.5 ml) on L cells	
	Series with interferon (200 U)	Series without interferon
TAI, unheated	2×10^{6a}	7.6×10^{7}
heated at 56°	2.2×10^{6}	8×10^{7}
75°	3×10^{6}	7.2×10^{7}
95°	1.4×10^{5}	8×10^{7}
Control interferon, 200 U	2.8×10^{5}	
challenge virus VSV		7.9×10^{7}

[a] Yield of VSV after the first cycle of multiplication.

Discussion. The amniotic and chorionic membrances contain a factor which blocks the action of interferon. The TAI has no direct effect on interferon *in vitro*, its action is mediated by the cells. Taylor (5) showed that when actinomycin and interferon are added simultaneously to the cells, the antiviral action of the latter is blocked. However, when actinomycin is added 4 hr after interferon, the antiviral action of interferon is not inhibited. Similar results were obtained with inhibitors of protein synthesis such as puromycin (6). This observation suggests that between 0 and 4 hr interferon derepressed an antiviral protein responsible for the "antiviral state." When added simultaneously with interferon to the cells TAI does not prevent the induction of the antiviral state by interferon. Consequently, antiviral protein derepressed by interferon can be synthesized in the presence of TAI. However, when TAI is added 4 hr after interferon, the antiviral action of the latter decreases considerably. It can be postulated therefore that TAI acts on a mechanism governed by the antiviral protein.

The chemical nature of TAI is poorly understood. The TAI does not dialyze or sediment after ultracentrifugation. It is resistant to heat inactivation and to low pH. TAI is also resistant to proteases and is present in the acido-soluble fraction after precipitation with 5% trichloroacetic acid. These properties exclude the possibility of TAI being either a simple protein or a nucleic acid. The physicochemical properties of the active moiety suggest the possibility of a complex molecule such as a glycoprotein. At present we can not state whether the integrity of cellular protein synthesis is necessary or not for the action of TAI.

The biological significance of this substance is also at present largely hypothetical. It seems possible that it may terminate an established antiviral state in nondividing cells.

As shown from previous studies *in vitro*, the antiviral state persists until cell division. The first generation after cell replication seems to be partially protected against viruses (7–8). The majority of the cells in different tissues (except cells of the hematopoietic system or the basic cell layer of mucosa) do not divide actively. When compared to the *in vitro* system, *in vivo* an established antiviral state should persist longer than it persists in reality. The TAI could contribute to decrease the antiviral state in cells which multiply slowly and restore cellular receptivity to viruses. Vilcek reported recently a factor extracted from fetal calf serum which has many similar properties to the antagonist here described. Further work will show whether it is identical to TAI or not (9).

Summary. Extracts of the amniotic and chorionic membranes contain a factor which blocks the action of interferon. The anti-interferon effect can be induced when the antiviral state mediated by interferon is already established. The tissue antogonist of interferon (TAI) is present in the acido-soluble fraction after precipitation with 5% trichloroacetic acid. It does not dialyze or sediment after ultracentrifugation. It is resistant to proteases, to heat, and to low pH. Although the biological role of this substance is unknown, it is postulated that it may contribute to terminate an antiviral state in the tissue and restore cellular receptivity to viruses.

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Received April 7, 1969. P.S.E.B.M., 1969, Vol. 132.