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Enhancement of Interferon Titers by Poly-L-Ornithine.* (32259)

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Protamine was shown by Amos(1) to enhance uptake of RNA by cultured chick cells. Subsequently, Ryser and Hancock(2) were able to stimulate uptake of albumin by tumor cells in culture with the addition of histones or basic polyamino acids. These findings suggested a possible means of enhancing the sensitivity of the interferon assay. If this were successful, the method would shed some light on the controversial question of the uptake of interferon by cells(3,4).

Materials and methods. Media. The growth medium consisted of Geys-Tris solution containing 5% lactalbumin hydrolysate, 5% fetal calf serum, 150 μg/ml potassium penicillin G and 250 μg/ml streptomycin sulfate. The same medium without fetal calf serum was used as diluting fluid. The agar overlay consisted of growth medium, 0.9% Bacto Noble agar, and 1:10,000 neutral red. *Poly-L-ornithine* (PLO) was employed as a crystalline powder, M.W. 140,000, obtained from Pilot Chemical Co., Watertown, Mass. *Chick embryo cells* were used as 24-hour, confluent monolayers in 60 mm plastic culture plates (Falcon) for interferon assays and virus titrations, and in 200 ml plastic culture flasks (Falcon) for production of virus and interferon. *Viruses.* Chickungunya virus (CV) from a mouse brain passage, and Sindbis virus grown in chick cells, were stored in sealed capillary tubes at −80°C; their titers on chick embryo cell monolayers were 2×10^7 and 2×10^8 plaque-forming units (PFU) per ml, respectively. *Interferon* was produced by inoculating chick cell monolayers with CV at a multiplicity of 0.1 PFU per cell. After incubation at 37°C for 48 hours, the tissue fluid was harvested and the virus inactivated by dialysis against 2 M KCl at pH 2.0 for 24 hours. The pH was readjusted to 7.4 by dialysis against Geys-Tris solution for 24 hours. Specimens of interferon were pooled and 1.0 ml aliquots were quick-frozen and stored at −80°C. For each experiment, the required number of aliquots were thawed and pooled, and portions were diluted with and without the indicated concentrations of PLO in the diluting fluid. The *standard interferon assay* was done in quadruplicate: growth medium was removed from plates with monolayers of chick embryo cells and replaced with 4 ml aliquots of diluting fluid or with a dilution of interferon. The plates were then incubated for 3 hours at 37°C. After the fluids were again decanted, 2 ml of an appropriate dilution of a Sindbis virus suspension, to contain 50-100 PFU, was delivered to each monolayer. Virus adsorption proceeded at 37°C for one hour, after which unadsorbed virus was removed and 6 ml of agar overlay was applied to each plate. Plaques were counted after incubation for 48 hours at 37°C. The interferon titer was expressed as the reciprocal of the dilution causing 50% reduction in plaques of Sindbis virus when compared to controls exposed to diluent alone.

Results. Although the primary interest in this study was on interferon, any concomitant effect of PLO on the virus challenge also had to be considered. To minimize any direct effect of PLO on the virus, the tissues were subjected to the fluids containing this polyamino acid only during the first hour of the standard 3-hour exposure to interferon. At

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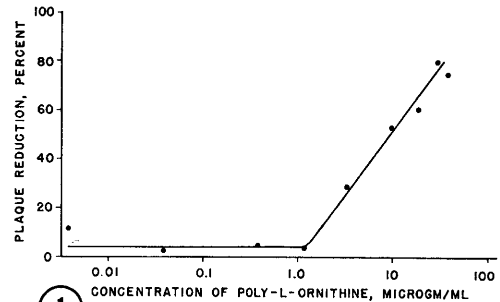

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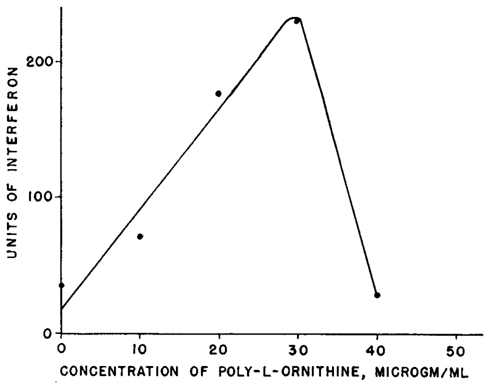

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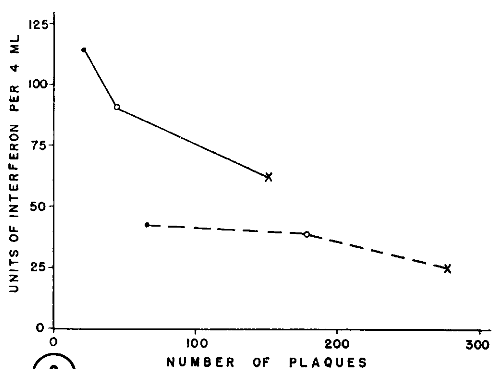

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FIG. 1. *Poly-L-ornithine (PLO) inhibition of Sindbis virus.* Monolayers on quadruplicate plates were treated for one hour with increasing concentrations of PLO. The plates were then washed twice and treated for 2 additional hours with diluent alone, after which they were challenged for one hour with 100 PFU of Sindbis virus. The percent reduction of PFU by various concentrations of PLO is shown as compared with PFU in similarly treated controls without PLO.

FIG. 2. *Poly-L-ornithine (PLO) enchancement of interferon titers.* Interferon assays were performed with PLO present during the first hour of treatment; the tissues were then washed twice and treated for additional 2 hours with the identical dilutions of interferon without PLO. The greatest dilution of interferon causing 50% plaque reductions as compared with controls without interferon is considered to be one unit.

FIG. 3. *Effect of number countable plaques on interferon titer.* Interferon titers were determined in the persence (———) or absence (- - -) of 10 μg/ml of poly-L-ornithine (PLO) during the first hour of treatment. Pairs of titrations (treated and not treated with PLO) were challenged with 3 dilutions of Sindbis virus which produced 66 (.), 178 (O) and 277 (X) PFU in the controls without PLO or interferon, and 20, 45, and 151 PFU, respectively in the controls without interferon but with 10 μg/ml PLO present.

that time the tissues were washed twice with diluent and further treated for 2 hours with identical interferon dilutions lacking PLO. The residual effect that PLO had on the plaquing of Sindbis virus challenge was documented in the following experiment: Sindbis virus was titered on tissue monolayers after they had been exposed to varying dilutions of PLO for 1 hour, washed twice and then exposed to diluent alone for 2 additional hours. In Fig. 1 the percent reduction of PFU in PLO-treated chick cells, as compared with cells treated with diluent alone, is plotted against the log of the PLO concentration used. Increasing concentrations of the PLO were thus seen to produce a reduction of plaque counts that was linear in the range of 3.3-30 μg/ml; 50% plaque reduction occurred at 10 μg/ml.

The effect of PLO on the interferon assay itself was determined by titering interferon in increasing concentrations of PLO, the virus control for each titration having the same concentration of PLO as the dilutions of interferon. The results are shown in Fig. 2. There was a linear increase in interferon titer with increasing concentrations of the PLO up to 30 μg/ml, after which it dropped off sharply. At the highest concentrations of PLO shown, the plaques were small and indistinct and at greater concentrations there was marked toxicity to cells and assay was no longer possible.

Toxicity to cells was further investigated by studying the effect of the continuous presence of 3-fold increases in concentrations of PLO on the ability of freshly trypsinized primary chick embryo cells to form a monolayer on stationary glass tissue culture tubes. When the initial suspension contained 4 ×

10^6 cells in 1 ml, a confluent monolayer was produced in 24 hours in the absence of PLO, and comparable confluent monolayers developed with PLO in concentrations up to and including 10 μg/ml. When the concentration of PLO was 30 or 90 μg/ml, debris was seen to adhere to the glass and monolayers of cells did not form. When initial suspensions of 10^6 cells per ml were used, only 90% confluent monolayers occurred after 96 hours in the absence of PLO; similar monolayers developed in the presence of PLO in concentrations up to 3.3 μg/ml, but with 10 μg/ml the cell sheet was only 50% confluent at 96 hours and a small amount of debris was already present. Greater concentrations of PLO had the same effect as was observed with the larger numbers of cells.

To investigate the unlikely possibility that the interferon-potentiating activity of PLO was secondary to the decreasing plaque numbers, 3 dilutions of Sindbis virus were used to challenge pairs of interferon titrations on chick cell monolayers, half of which had been exposed to 10 μg/ml PLO during the first of the 3 hours of treatment. The results are depicted in Fig. 3 in which the interferon titers are plotted against the numbers of plaques forming in their respective controls lacking interferon after inoculation of the 3 dilutions of virus. The curves show a parallel trend but the titers were higher in the presence of the PLO for each of the 3 virus inocula used.

Discussion. Amos(1) and Amos and Kearns (5) found that protamine sulfate would enhance the uptake of P^{32} labeled bacterial RNA and DNA into chick cells. Subsequently, Ryser and Hancock(2) showed that histones and several basic polyamino acids stimulated the uptake of radio-iodinated or fluorescein-labeled albumin by cells of Sarcoma 180; the most effective polyamino acid they used was PLO, hence this substance was chosen for the present study. Others have shown enhancement of the titer of mature virus after infection of cells with RNA prepared from poliovirus, Smull and Ludwig(6) using protamine sulfate, Pagano and Vaheri(7) employing the polycation diethylaminoethyl dextran, and Koch, Quintrell and Bishop(8) utilizing PLO. Basic macromolecules increase pinocytosis and phagocytosis by macrophages and, as suggested by Ryser and Hancock(2), may show similar membrane effects on the fibroblast and epithelial tissues used in such studies. The possibility that the great positive charge on these molecules at neutral pH is responsible for an alteration in negatively charged cell membranes warrants further investigation. In the present study, the pH was not changed appreciably by the concentrations of PLO used.

Attempts to demonstrate uptake of interferon by cells have yielded conflicting results. Buckler, Baron and Levy(3) were able to induce antiviral activity in cells without a detectable or significant loss of the applied interferon activity from the overlying medium. On the other hand, Ke, Armstrong, and Ho (4) did demonstrate a loss of biological activity of interferon preparations when exposed to cells and also showed that the volume as well as the concentration of the interferon preparations influenced the antiviral activity induced in the cells; they maintained that volume would be irrelevant if no interferon were adsorbed during the induction of the antiviral state. The results reported here show that the sensitivity of a standard interferon assay can be increased up to 7-fold with increasing concentrations of the basic polyamino acid used, namely PLO. This is interpreted as showing that this basic substance facilitated the uptake of interferon by cells and thereby revealed an interferon effect at levels that otherwise would not be detected. The mechanism responsible for the postulated effect is not known, but a reversible toxic effect on the cells may be involved. These findings would tend to support the results reported by Ke and co-workers(4).

Summary and conclusions. The sensitivity of a standard interferon assay was increased by exposure to poly-L-ornithine during interferon treatment. The enhanced titers thus demonstrated are interpreted as indicating an increased interferon uptake by the cells.

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Effects of Exercise on Plasma and Tissue Levels of Lactate Dehydrogenase and Isoenzymes in Rats. (32260)

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In recent years there has been a growing interest in the effects of stress, especially physical exertion, on untrained individuals. The rat has proved to be a useful animal for studying these effects in the laboratory. Marked rises in serum lactate dehydrogenase (LDH) activity and in other enzyme levels have been reported in untrained rats subjected to different types and severity of exercise (1-5). No explanations were given in these studies for the high levels of serum LDH seen in the control animals nor for the marked variation in levels in both the control and exercised groups(1-3). Studies in our laboratory showed similar discrepancies and investigations were undertaken which resulted in the elucidation of several factors contributing to high control values and variability between determinations. To control these factors, a modified method was evolved for collecting and preparing rat blood for LDH analysis. This method was then employed along with an electrophoretic procedure for determination of LDH-isoenzymes, recently developed by one of the authors(6), in order to better evaluate serum and tissue LDH changes resulting from exercise in rats.

Experimental procedure. Twenty untrained male rats of the Walter Reed strain, weighing 150 to 250 g, were randomly divided into an exercised group and an unexercised control group. Both groups were fasted overnight prior to the procedure. The exercise group was subjected to 4 hours of swimming with a 5-minute rest every 30 minutes, using a 30 gallon, 52 × 52 × 92 cm tank of water at 30-32°C temperature. Within 20 minutes after completion of exercise, the animals were anesthetized with ether, the abdomen opened, blood was removed from the inferior vena cava using a plastic syringe, and placed in a plastic tube containing heparin (50 μg/3 ml blood). Immediately after removal it was centrifuged for 3 minutes in a microfuge (12000 rpm), to separate the plasma which was subsequently analyzed for LDH and isoenzymes.

Following the collection of blood, tissue samples were excised from the liver, cardiac ventricles and thigh muscle, and the animal was sacrificed. These tissues were rinsed in saline, blotted and weighed. Ten per cent tissue homogenates were prepared in a glass homogenizer with buffered saline pH 7.5. The homogenates were then centrifuged at 15000 rpm for 10 minutes and the supernatants were analyzed. Histological sections were prepared from the excised tissues fixed in 10% Formol-Ringers solution and stained with H & E, PAS, and Oil Red O. Histological sections were also prepared from other animals 3 days post exercise.

Red blood cell and platelet preparations were made by centrifuging heparinized blood from control rats at 600 rpm for 10 minutes. An aliquot was removed from the supernatant