TABLE I.

Flask No.	Location	Vol, ml	Glucostat determin'n, μg	Predicted conc. on basis of free distribution	% of total glucose recovered
1	Inside bag	7	28	27.5	98
	Outside "	50	27		
2	Inside "	7	42	42	98
	Outside "	50	41		
3	Inside "	7	31	27.5	100
	Outside "	50	28		
4	Inside "	7	46.8	42	105
	Outside "	50	44		
5	Inside "	7	28	27.5	101
	Outside "	50	29		

μg of glucose/ml in Flask 1 and 38.4 μg of glucose/ml in Flask 2. This would have been the case if the glucose bound to insulin were *not* liberated when the solution was deproteinized. In this event only 89% of the glucose would have been recovered from Flask 1 and 92% from Flask 2 instead of the observed 98%.

If one mole of glucose were bound to insulin and liberated upon deproteinization, then there would have been an increase of 1 micromole of glucose within the bag in both Flasks 1 and 2. In this case there would have been 50.9 μg/ml instead of the observed 28 μg/ml in Flask 1 and 64.1 instead of 42 μg/ml in Flask 2.

All of the initial glucose was recovered, therefore, deproteinization liberated any glucose which may have been bound. Since there was no disparity between concentration of glucose inside and outside the bag, it is assumed that no glucose was bound. It would be inconsistent with the initial postulate to propose that less than one mole of glucose/mole of insulin were bound. In the light of these experiments it is assumed that, at physiologic ionic strength and pH, insulin does not bind with glucose.

Summary. Insulin does not retard distribution of glucose through a semipermeable membrane under the conditions of this experiment. Conditions in different media may show different results. It was decided not to investigate the initial premise further since the conditions of these experiments were similar to the pH and salt concentration encountered *in vivo*.

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Development of a Strain of Psittacosis Virus Partially Resistant to 5-Fluorouracil in a Tissue Culture System.* (26715)

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The development of resistance to chemotherapeutic agents by psittacosis virus has been described(1-6). The present paper describes the development of resistance to 5-fluorouracil (FU) by strain TTF psittacosis virus in a tissue culture system.

Materials and methods. "Wild-type" vi-

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TABLE I. Comparison of Sensitivity to 5-Fluorouracil (FU) between Wild-Type and FU-Resistant Strain of Psittacosis Virus.

Virus phenotype	Exp.	Virus units/ml produced in presence of FU at a concentration indicated below (μg/ml) 0 (control)	1	2	3	4
"Wild-type"	1	1.1×10^7	4100	<10	—	—
	2	3.3×10^7	1700	<10	—	—
	3	1.1×10^7	2700	<10	—	—
FU-resistant strain	1 (6th pass.)	1.0×10^7	7.5×10^5	5.5×10^5	—	300
	2 (7th ")	2.3×10^7	1.2×10^6	4.5×10^5	—	—
	3 (10th ")	1.6×10^7	1.6×10^6	5.7×10^5	1.1×10^4	900

— = Not tested.

rus stock. The 34th passage of strain TTF psittacosis virus(7) in cultures of the McCoy cell line(8) represented the wild-type virus stock. The latent period of TTF virus in McCoy cell cultures was about 17 hours at 37°C and thereafter newly synthesized infective virus increased in number rapidly. Maximum yield of virus was reached by 48 to 72 hours after infection to a level between 5×10^7 and 10^8 infective units per ml. The replication pattern and cytochemical changes associated with virus multiplication were reported elsewhere(9-11).

Host cell. A cellular derivative of human synovial tissue (strain McCoy) was propagated on coverslips in Leighton tubes, or in milk-dilution bottles. The nutrient medium consisted of Mixture 199 with 0.5% lactalbumin hydrolysate, 5% heat-inactivated calf-serum and 0.1 mg per ml streptomycin. The pH was adjusted to 7.2 by sterile sodium bicarbonate.

Infective virus units were assayed at 24 hours after inoculation by the "infected cell count method"(7,12).

5-Fluorouracil‡ FU was dissolved in distilled water (1 mg per ml), sterilized by Selas filtration and stored at −20°C. Graded amounts (25 μg to 5 μg/ml) were dissolved in nutrient medium and tested for effect on normal cells. Significant cytotoxicity was not observed at concentration of 25 μg/ml or less.

Results. Sensitivity of TTF virus to FU. Inhibitory action of FU on maturation of psittacosis virus was determined according to the procedures described in preceding experiments(13). Virus was inoculated, with a multiplicity of infection of 3, onto monolayer cell sheets on coverslips in Leighton tubes (approximately 5×10^5 cells per tube). Thus most of the cells were infected. After an interval of one hour, inoculated tubes were washed once and 1.5 ml of nutrient medium with graded dosages of FU was added to each. The infected cultures were then incubated at 37°C for 36 hours, during which time maturation of virus in FU-free control cultures was virtually complete, without extensive cell destruction. All cell cultures were then washed twice with fresh FU-free medium, and then with 2 ml of the same medium, each was subjected to repeated freezing. Infective virus units of such treated cultures were assayed by the infected cell count method.

The results of this experiment are shown in Table I and Fig. 1. FU inhibits maturation of the wild-type TTF virus completely at 2 μg per ml (1.5×10^{-5}M). There is a linear dose-response relationship between concentration of analogue in the nutrient medium and log units of virus produced (Fig. 1).

Isolation of FU-resistant viral strain. Isolation of a virus strain which was resistant to FU was carried out by repeated selections and multiplications, as follows: Three ml of the wild-type virus (3×10^8 infective units) was inoculated onto a bottle culture of McCoy cell. After absorption of one hour, 10 ml of the medium containing FU at 0.5 μg/ml was added. After incubation for 2 days at 37°C, the cell culture was washed with Mixture 199 and replaced with 10 ml of FU-free medium. The culture was twice

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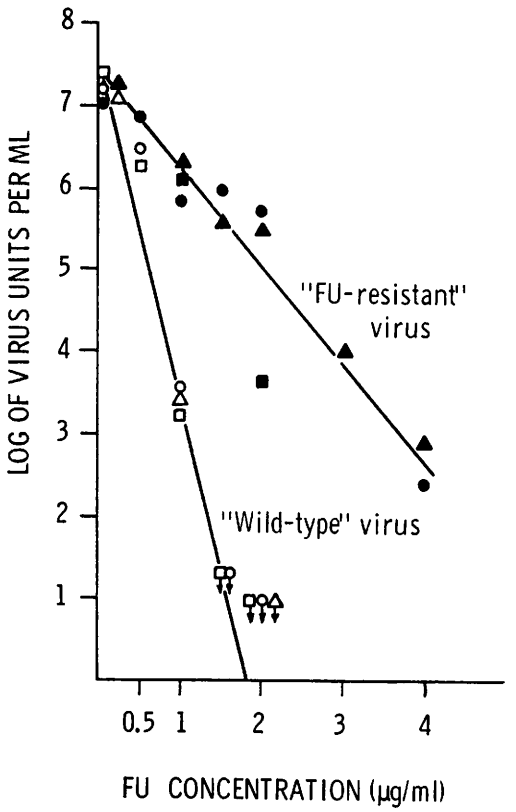

FIG. 1. FU-sensitivity of "wild-type" and FU-resistant psittacosis viruses. Virus levels produced in presence of FU are plotted as functions of analogue dosages. Symbols with downward arrows represent that the infective virus units were below a titer indicated by each point itself.

frozen and assayed. The cell culture thus prepared contained 5×10^5 infective units per ml. Test of FU-sensitivity showed that the surviving virus was almost as sensitive to FU as the wild-type virus. This virus was inoculated into a bottle containing fresh cells (without FU) in which the propagating virus caused the cells to lyse after several days. The virus titer in the resulting virus stock (grown in FU-free nutrient medium) exceeded 2×10^7 per ml. The latter virus stock was again inoculated into another bottle of McCoy cells and incubated with medium containing 0.5 μg FU/ml. After several cycles of selection-and-multiplication of virus as described above, virus acquired the capacity to mature in medium containing a borderline concentration of 2 μg FU/ml. Table I and Fig. 1 show the results of FU-sensitivity tests carried out with the 6th, 7th and the 10th passages. The FU-resistant strain produced more than 10^5 units/ml of medium containing 2 μg FU/ml. Wild type virus was not produced in cultures which contained 2 μg FU/ml. It is apparent that the virus acquired some tolerance to FU during the serial passages in small amounts of the compound. In spite of further efforts, however, resistance of TTF virus to FU beyond the 2 μg/ml could not be demonstrated.

To determine the stability of the FU-resistance acquired by TTF virus, it was passaged through 5 series of cultures with FU-free medium, and again was propagated in medium with FU. A significant loss of FU-resistance was not observed. Thus, it may be concluded that the low level of FU-resistance of TTF virus was due to certain mutation(s) in virus genome rather than due to adaption.

Discussion. Cohen(13) and Heidelberger *et al.*(14,15) indicated that FU interfered with both RNA and DNA biosynthesis through its incorporation into RNA or by blocking the methylation reaction leading to formation of DNA-thymine, respectively. In addition, Skold(16) pointed out a direct inhibition of the pyrimidine nucleoside phosphorylase due to FU in the Ehrlich ascites tumor cells. Thus FU inhibits the synthesis of both RNA and DNA through at least 3 different processes.

Failure to isolate a virus strain completely resistant to FU may be attributed to the multiplicity of sensitive points by FU on biosynthesis of nucleic acid. The explanation that the acquired FU-resistance of psittacosis virus may be due to acquisition of FU-resistance by the host cell is readily discounted because virus was passaged always to fresh cells from cultures containing virus and killed cells.

The development of resistance to FU by psittacosis virus supports the claim that this microorganism possesses specific enzyme systems for synthesizing viral materials, especially for biosynthesis of viral nucleic acid, which are, at least in part, different from those of host cell itself.

Summary. A strain of psittacosis virus (TTF) developed resistance to 5-flourouracil (FU) after serial passages of the original FU-sensitive virus in tissue culture cells with small concentration of FU in the medium. The FU-resistant strain of TTF virus multiplied and produced more than 10^5 infective units per ml in presence of 2 μg FU/ml, at which level FU suppressed the original wild-type TTF virus completely. Reversion of character from FU-resistance to FU-sensitivity following serial passages in FU-free medium was not observed. This suggests that the acquisition of FU-resistance is due to certain gene mutation(s), rather than to enzymatic adaptation.

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Comparison of Assays for Mengovirus and Reovirus-3.* (26716)

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Reoviruses were recently recognized as a group distinct from enteroviruses and other well known groups of animal viruses(1). In view of the susceptibility of weanling mice to these viruses(1), it appeared reasonable to determine whether reoviruses would grow and produce cytopathic effects in L-cells, a well defined cell line originally derived from mouse connective tissue. This paper describes the conditions for plaque assay of reovirus-3 and compares these conditions with those for the plaque assay of Mengovirus in L-cells, a virus which has been studied in this laboratory in recent years.

Materials and methods. L-cell, strain 929, originally obtained from Microbiological Associates, Inc., was cured of PPLO infection with kanamycin(2) and maintained free of PPLO by continued use of kanamycin in the cell stock cultures. The medium for stock cultures consisted of Eagle's minimum essential medium (MEM)(3) supplemented with 5% horse serum. Serum from a single selected horse was used, since sera from some horses are cytotoxic. The following antibiotics were used in the stock cultures: penicillin (500 units/ml), streptomycin (0.5 mg/ml), mycostatin (25 units/ml), and kanamycin (0.5 mg/ml). When stock cells were plated for any biological studies or plaque assays, the same nutrient medium was used, but only streptomycin and penicillin were present.

Mengovirus was obtained from Dr. M. Theiler as a frozen mouse brain suspension,

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