TABLE IV. Stability Constants (K) for Complexes of DFOM with Several Metallic Ions.

Ion	K
Mg^{++}	10^4
Ca^{++}	10^2
Sr^{++}	10
Co^{++}	10^{11}
Ni^{++}	10^{10}
Cd^{++}	10^3
Zn^{++}	10^{11}
Fe^{+++}	10^{31}

General equation: metallic ion of valance n : M^{+n} DFOM as tribasic acid : AH_3 : $M^{(+n)} + AH_3 = MAH_{3-n} + nH^{(+1)}$

$K = ([M \cdot AH_{3-n}])/([M^{(+n)}] \cdot [AH_{3-n}{}^{(-n)}])$

without insulin. This fact rules out 3 of the above hypotheses, *i.e.*, those assuming its effect on the insulin molecule. If the first hypothesis were correct, that the effect of the drug is due to the removal of a metal ion from the membrane, the high affinity of DFOM for iron in comparison with other metal ions, as shown in Table IV(5) and the low dosage necessary to detect its activity has led to the assumption that iron might be the ion metal removed from the cell membrane in order to increase glucose permeability.

These data also suggest that the increased glucose tolerance observed in patients with hemochromatosis treated with DFOM is related to increased peripheral glucose utilization induced by the drug.

Summary. DFOM increases glucose uptake by the rat epididymal fat pad *in vitro*. This effect is also evident when insulin is present in the incubation medium. It seems reasonable to assume that the improved glucose tolerance shown by patients with diabetes and hemochromatosis during DFOM treatment is due to increased peripheral utilization.

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Persistence of Active Interferon in Cells Washed After Treatment With Interferon. (30959)

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Experiments performed at 37°C in which puromycin(1-3) was used to block the action of interferon suggested that interferon could not be removed from cells by extensive washing and led to the work described below to demonstrate its persistence in treated cells.

Materials and methods. Virus. The western equine encephalitis (WEE) virus used was grown at 37°C in an atmosphere of 4% CO_2 in air in monolayers prepared from trypsinized chicken embryo, as previously described(1), and was assayed by the plaque method. The virus growth medium consisted of 0.1% lactalbumin hydrolysate, balanced salts and 2% calf serum. The same medium supplemented with 0.9% agar was used for virus assay. Each cell monolayer was inoculated with 0.5 ml of virus which contained 9×10^7 plaque forming units (pfu), providing a virus-to-cell ratio of approximately 25. Virus was harvested after cells had been disrupted by a cycle of freezing and thawing.

Interferon. The interferon used was prepared from allantoic fluid infected with influenza B virus, and was partially purified by 2 cycles of zinc precipitation followed by dialysis for 24 hours against 0.9% NaCl and for another 24 hours against 0.01 M phosphate

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buffer at pH 6.0(4). The partially purified interferon was stored in 1.0 ml amounts at —20°C, thawed before use, and not refrozen after thawing. Interferon was assayed by the method of Wagner(5), according to the following procedure. Serial dilutions of interferon and approximately 50 pfu of virus were added to plated chicken embryo cells which were then incubated at 37°C for one hour and overlaid with the same agar medium used for virus assay. The dilution which reduced the plaque count 50% was the endpoint. The interferon preparation used in the experiments summarized in Table II had a titer of approximately 100 units per ml. The preparation used in all other experiments had a titer of 1800 ± 350 units per ml, as determined by 6 separate titrations over a period of almost 1 year. *Tissue cultures* used in these experiments were prepared from embryonated eggs of chickens free of RIF (a factor inducing resistance to Rous sarcoma virus).

Results. The experiments that led to the present investigation consisted of exposing cells at 37°C to 0.1 ml of interferon (180 units) in 0.9 ml of medium 199 containing 10 γ of puromycin to inhibit the action of interferon. At the end of the adsorption period (4 hr), the monolayers were washed 3 times with 5.0 ml of PBS (phosphate buffered saline) per wash. Some monolayers were immediately challenged with virus by adding 0.5 ml of virus to each monolayer. After 1 hour at 4°C, to permit attachment of the virus, the monolayers were again washed 3 times, this time with 10.0 ml of PBS per wash, overlaid with 4.0 ml of virus growth medium, and again incubated at 37°C. The remaining monolayers were overlaid with 1.0 ml of medium 199, incubated at 37°C for another 4 hours, and then also challenged with virus. After 20-24 hours, the virus was harvested. The results (Table I, Part A) indicated that following exposure to interferon in the presence of puromycin and repeated washing to remove both agents, the resistance of the cells (as measured by virus yield) increased on further incubation in the absence of interferon. However, the increase in the virus-resistance was sensitive to both actinomycin D and puromycin, as is the case with interferon (Table I, Part B). This result suggested that the repeated washing had not removed all of the interferon to which the cells had been exposed and that the residual interferon could make the cells resistant to virus growth once the puromycin was no longer present.

TABLE I. Cells Infected with Virus After Exposure to Interferon (180 Units) in Presence of Puromycin (10γ) and Washing to Remove Both Agents.

Cell treatment	Virus* yield (% of control)
A. Development of Resistance to Virus Synthesis	
None (control)	100.00
Interferon alone	.005
Interferon + puromycin:	
Infected immediately after washing	.60
Infected 4 hr after washing	.05
B. Effects of Actinomycin D and Puromycin on Development of Resistance to Virus Synthesis†	
None (control)	100.00
Interferon alone	.06
Interferon + puromycin:	
Infected immediately after washing	.92
Infected 4 hr after washing	.12
Actinomycin D (1γ) added after washing	.45
Puromycin (50γ) added after washing	.55

* Western equine encephalitis virus.

† Monolayers were washed after exposure to interferon in presence of puromycin at 37°C for 4 hr. One plate was infected immediately. To the other plates was added medium 199 alone or containing actinomycin D or puromycin, and they were incubated at 37°C for 4 additional hr before infection. The control exposed to interferon alone was also incubated at 37°C for 4 hr.

In the next 2 experiments a temperature of 4°C was used in place of the presence of puromycin to provide a condition which would permit adsorption of the interferon, but would prevent its functioning(6,7). Test monolayers were exposed to 0.1 ml of interferon, whose titer was approximately 100 units per ml, and 0.9 ml of medium 199 at 4°C for 2, 4, or 15.5 hours. Controls were handled similarly and included monolayers exposed to interferon at 37°C and monolayers that were not treated with interferon. All monolayers were then washed 3 times with 10.0 ml of cold PBS per wash. Some of the test monolayers and all the control monolayers were challenged with

TABLE II. Cells Infected with Virus After Exposure to Interferon (0.1 ml containing approximately 100 units per ml) at 4°C. Separation of interferon adsorption from interferon action.

Exp No.	Adsorption (hr at 4°C)	Action (hr at 37°C)	Virus* yield (% of control)
1	Control	No interferon	100.0
	0	4	2.3
	4	0	82.0
	4	4	9.0
2	Control	No interferon	100.0
	2	0	86.0
	4	0	36.0
	15.5	0	93.0
	2	4	12.0
	4	4	15.0
	15.5	4	8.0
	0	2	4.0
	0	4	.5
	0	15.5	.3

* Western equine encephalitis virus.

virus immediately after washing. The other test monolayers were first overlaid with 4 ml of either virus growth medium (Exp. 1) or medium 199 (Exp. 2), and incubated at 37°C for 4 hours before they were challenged with virus. One hour at 4°C was permitted for attachment of the virus to all the monolayers.

The results of this experiment agreed with the findings of Lindenmann *et al*(6), and Vilcek and Rada(7). The monolayers exposed to interferon at 37°C were more virus-resistant than those exposed at 4°C, but when the latter were washed and then incubated at 37°C, their resistance increased (Table II). This increase in virus resistance at the higher temperature is explained most simply by the retention by the cells of some of the interferon to which they had been exposed, which became active when the temperature was favorable.

The first two attempts to demonstrate the presence of this residual interferon were unsuccessful, probably because the approximately 180 units of interferon to which the cells were exposed was too little. In the first experiment, the treated monolayers were incubated for 5-6 hours at either 37°C or 4°C, washed 3 times, frozen and thawed once to disrupt the cells, and assayed for virus inhibitor. No inhibitor was found, which agreed with the results obtained by Wagner(5). In the second unsuccessful experiment, an attempt was made to dissociate the interferon from the cells by lowering the pH to pH 2 by treatment with 0.1 M HCl for 15 minutes at 4°C; after centrifugation, no inhibitor was found in the supernate.

However, the presence of virus inhibitor could be demonstrated when the monolayers were exposed to a greater quantity of interferon. In this experiment, monolayers were exposed to 0.5 ml of interferon containing approximately 900 units, and 0.5 ml of medium 199 containing 1% cow serum for 5 hours at 4°C. Control monolayers were exposed to 1.0 ml of the same medium (but no interferon) at the same temperature for the same period. All monolayers were then washed 3 times with 10.0 ml of cold PBS per wash, and assayed for virus inhibitor. In the first part of the experiment, this was done by adding to each plate of a set of test and control plates 1.0 ml of the same medium described above, freezing and thawing the plates at 4°C, and assaying the cell debris. In the second part of the experiment, 2.0 ml of the same medium and sufficient 0.1 M HCl to adjust the monolayers to pH 2 were added to each plate of a set of test and control plates, and these were held at 4°C for another 15 minutes, frozen and thawed at 4°C, centrifuged to remove cell debris (in a table model Servall angle-head centrifuge at 4500 rpm for 10 minutes), and the supernate was assayed for virus inhibitor. Approximately 10 units of an inhibitor were found in both the disrupted cell preparation and the supernate of monolayers treated with interferon but not in those of the control monolayers (Table III). This virus inhibitor was presumed to be interferon.

Discussion. In the experiments demonstrating the presence of an inhibitor of virus synthesis, presumably interferon, in cells washed after exposure to interferon at 4°C (Table III), the wash fluids were evacuated by means of a pipette connected to a vacuum line. If as much as 0.5 ml of fluid had remained after each removal, the 3 washes with 10 ml of fluid per wash, followed by overlay with 1 ml of fluid, would have reduced the concentration of an evenly distributed solute by a factor of 24,000. Despite this dilution factor, at least 1% of the interferon to which

TABLE III. Cells Infected with Virus After Exposure to 900 Units of Interferon at 4°C and Washing to Remove Interferon: Recovery of Residual Interferon.
(Cells assayed for virus inhibitor were either disrupted or in form of acid-treated supernate.)

	No. of virus* plaques per assay plate treated with			
	Disrupted cells (0.1 ml)		Supernate (0.2 ml)	
Dilution added	Interferon-treated	Control	Interferon-treated	Control
Undiluted	13, 9 (11)	35, 43 (39)	6†, 10 (8)	62, 50 (56)
Diluted 1:3	38, 34 (36)	51, 45 (48)	57, 56 (56)	56, 66 (61)
" 1:10	56, 52 (54)	49, 66 (58)	41, 77 (59)	52, 55 (54)
" 1:30	39, 50 (45)	47, 47 (47)	50, 48 (49)	65, 56 (60)
Control	37, 48 (45)			
Interferon titer (approximate)	1 unit per 0.1 ml		1 unit per 0.2 ml	
Interferon recovered (approximate)	10 units per plate		10 units per plate	
Control titrations of interferon used (approximate)	3,000 units per ml		(1,500 units after adjustment to pH 2)	

* Western equine encephalitis virus.

† Pinpoint colonies.

the cells were exposed could be recovered.

The data presented in Table I suggest that such tightly-bound interferon is also present in cells after exposure to interferon at 37°C. Other investigators(8,9) have noted the difficulty of washing out interferon and Friedman and Sonnabend(3) observed that the resistance to virus infection in cells treated with interferon continued to increase after removal of the interferon. This effect might be due to residual interferon, which could continue to act over a long period.

The experiments involving cell treatment with interferon in the presence of puromycin (Table I) were initially designed to investigate the role of RNA and protein induced by interferon(1,2,3,10) in the development of resistance to virus infection. The presence of puromycin would inhibit protein synthesis but not RNA synthesis and would permit the RNA induced by the interferon to accumulate. When the puromycin was removed, this RNA would act as a messenger for protein synthesis. The cells would then become resistant to virus infection without the addition of more interferon. As pointed out above, the resistance that developed was most probably due to interferon that could not be removed by washing.

However, the fact that no resistance, or very little, developed in the presence of actinomycin D (Table I, Part B) suggests that no RNA induced by the interferon was accumulated and, therefore, such RNA must have a relatively short half-life (less than 4 hours). The persistence of interferon-induced resistance(11) would then appear to depend either upon the existence of a virus-inhibiting protein with a relatively long half-life or upon the continued presence of interferon itself.

Summary. An inhibitor of virus action obtainable from cells washed after treatment with interferon at 4°C, but not from untreated cells, was presumably interferon. The quantity recovered amounted to approximately 1% of the interferon to which the cells had been exposed. Persistence of interferon could be demonstrated in cells extensively washed after exposure to interferon at 37°C by exposing the cells to interferon in the presence of puromycin (10γ per ml) to block the

virus inhibitory action of interferon. When the washed cells were again incubated at 37°C before they were infected with virus, they became resistant to virus synthesis without further exposure to interferon. The virus resistance developed by cells under these conditions was blocked by actinomycin D. The significance of these results is discussed in terms of the persistence of resistance to virus infection after interferon treatment and the probable half-life of interferon-induced RNA.

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Effect of Pentobarbital Anesthesia on Plasma Half-Life of Indocyanine Green in Beagles.* (30960)

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Indocyanine green (ICG) has been suggested by several investigators as an agent useful in evaluation of hepatic activity(1-3). Several reports have appeared wherein the plasma half-life (T-½) of this tricarbocyanine dye has been determined while the animals were anesthetized(1,3). Since various investigators have reported that pentobarbital may interfere with the hepatic extraction of ICG(1,3), this investigation was undertaken to determine the effect of pentobarbital anesthesia on the plasma T-½ of ICG in beagles.

Method. ICG clearance was determined in purebred healthy beagle dogs of either sex, ranging in weight from 4.5 to 13.4 kg and between 1 and 2 years of age, by the method of Ketterer *et al*(1). After overnight fasting, ICG (2.5 mg/ml aqueous solution) was given intravenously at a dosage of 0.5 mg/kg. Two or three heparinized blood samples were taken from the jugular vein of each animal at intervals between 2 and 10 minutes after ICG injection; exact time of blood sampling was recorded in each instance. Plasma was separated following centrifugation at 3000 rpm for 15 minutes. A pre-injection blood sample was taken for blank determinations and standard preparation. Plasma concentration of ICG was determined by diluting 1 ml of plasma with 2 ml distilled water and measuring the optical density at a wave length of 805 mμ on a B & L Spectronic 20 adapted to the infrared absorption range.

Plasma T-½ and fractional clearance were determined as reported previously(4).

* In the manuscript entitled "Hepatic Clearance of Indocyanine Green in the Beagle" by Vogin, *et al.*, Proc. Soc. Exp. Biol. and Med., 1965, v119, 570, an error appears in Table II for the values of volume of distribution, plasma clearance and estimated hepatic blood flow. Since the animals employed in this study are no longer available, it is not possible to determine exact values for these 3 parameters. However, studies performed in other animals indicate that if the value given in the text is multiplied by the factor 0.534, an estimate of the correct value for the above parameters can be obtained. (Example, Volume of Distribution for Beagles: 85 ml/kg × 0.534 = 45.4 ml/kg.)