mental diets 23 days, while the data in Table II are considerably smaller, since they were obtained from rats which had been on the diets for only 17 days. In any case, the effect of the dietary deficiencies studied on pancreatic zinc were consistent in each study.

Whether the increased zinc levels observed in thiamine and vitamin B_6 deficient rats are simply a reflection of the inanition which accompanies the production of these deficiencies states cannot be determined from these data.

Compared to the effects of vitamin B_6 deficiency and caloric restriction on zinc content of the tissues examined, their effects on tissue magnesium and potassium levels were either not significant or of relatively small magnitude.

It is of some interest to speculate whether the increased tissue zinc levels produced by nutritional deficiencies in these studies might serve some physiological purpose. Insulin plays a major role in glucose transport and is a primary hormone involved in lipogenesis and the deposition and retention of fat in tissue.

To protect animals in a wide variety of metabolic states including nutritional deficiencies, it seems likely that control mechanisms regulating the activity and availability of insulin must exist in animals. It has been shown that 0.048 μg of zinc/ml of Krebs–Ringer–bicarbonate buffer containing 3 mg of glucose and 0.5 mU of crystalline insulin inhibits the lipogenic activity of isolated rat epididymal adipose tissue. This inhibition increased as the concentration of zinc increased (3). In the present studies the mean serum zinc values ranged from 1.19 to 4.18 μg/ml. Fasting and deficiencies of vitamin B_6 and B_1 resulted in increased tissue levels of zinc which were most marked in the pancreata. Whether these changes in zinc concentrations are reflected in altered insulin metabolism remains to be studied.

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Reovirus Type 2: Production of and Sensitivity to Interferon in Human Amnion Cells (RA)* (32912)

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Several RNA- and DNA-containing animal virions have been found capable of inducing the production of viral inhibitors called interferons in different animal and cell species. Among these virions are myxoviruses (1), arboviruses (2), papovaviruses (3), herpes viruses (4) and picornaviruses (5). The present report will describe the production of an interferon-like substance in a stable line of human amnion cells (RA) by the double-stranded RNA containing reovirus type 2.

After the completion of these studies a report appeared describing the production of interferon under *in vivo* conditions by the isolated double-stranded RNA component of reovirus type 3 (6).

Methods and Materials. Cell cultures. Monolayer cultures of RA (human amnion),

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BSC-1 (green monkey kidney) and mouse L cells were serially maintained and used as described previously (7).

Virus. Reovirus type 2 and vaccinia virus were maintained by passage in RA and HeLa cells, respectively. The titer for reovirus type 2 in BSC-1 cells was 1.8×10^8 infectious units (IU)/ ml and for vaccinia virus in RA cells was 5×10^6 plaque forming units (pfu)/ ml. Vesicular stomatitis virus (VSV) was obtained from Dr. Sam Baron of the NIH and its titer in HeLa cells was 4.8×10^6 pfu/ ml.

Induction of viral inhibitor. Viral inhibitor preparations were obtained by infecting monolayers of RA cells with reovirus type 2 at an exposure multiplicity of about 5. After incubation at 37°C for 48 hours, the infected cultures were frozen and thawed 2× and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant fluid was acidified to pH 2 and kept at 4°C for 24 hours. It was previously determined that such acid treatment of reovirus resulted in the inactivation of greater than 98% of virions. In order to remove any residual viable virions, the acidified preparations, after adjustment of the pH to 7.4, were subjected to 2 cycles of high-speed centrifugation at 45,000 rpm for 1.5 hours. The final supernatant fluids were found free of viable virions and possessed antiviral activity. Although the potency of such preparation varied, antiviral activity was always present.

Assay of antiviral activity. The assay for antiviral activity of the inhibitor preparations was based on the suppression of cytopathic effect (CPE) according to the method of Sellers and Fitzpatrick (8). Briefly, tube monolayer cultures of RA cells were exposed to various concentrations of the inhibitor for 18–24 hours at 37°C. After removal of the inhibitor by 4 rinses with GKN (0.025 *M* glucose and 0.005 *M* KCl in saline), the cell cultures were challenged with 3,000 pfu of VSV and reincubated at 37°C. Scoring of the cultures was done when the virus controls exhibited 100% CPE (4+). The dilution of inhibitor that provided approximately 50% (2+) protection to the cell monolayer was considered to contain 1 unit of antiviral activity. The crude preparations of the inhibitor when tested in the above manner contained 15 to 30 units/ml of antiviral activity.

Results. Characterization of the antiviral inhibitor. To learn whether the viral inhibitor present in the supernatant fluid from RA cell cultures infected with reovirus type 2 could be classified as an interferon, several of its biologic and physico-chemical properties were determined. The inhibitor was found to be: (i) stable at pH 2 for 24 hours, (ii) irreversibly adsorbed to cells, (iii) not sedimentable at 45,000 rpm for 1.5 hours, (iv) not inactivated by reovirus type 2 neutralizing antibody, and (v) not toxic to the cells even when the cell cultures were exposed to the undiluted inhibitor preparation for 24 hours (Table I). The following additional critical criteria were examined.

TABLE I. Properties of the Viral Inhibitor Induced in RA Cells by Reovirus Type 2.

Treatment	Effect
pH 2	Stable
Ultracentrifugation (45,000 rpm for 1.5 hrs)	Not sedimented
Cell-bound	Positive
Trypsin (0.2 mg/ml for 1 hour at 37°C)	Activity completely lost
Actinomycin D (1 μg/ml)	Action inhibited
Host specificity:	
RA cells	Positive
BSC-1 cells	Positive
Mouse L cells	Negative
Effect on different viruses:	
Vaccinia	Positive
Reovirus type 2	Positive
Vesicular stomatitis virus	Positive
Toxicity to cells (undiluted inhibitor for 24 hrs)	Negative
Reovirus type 2 antiserum	Negative

Chemical composition. Treatment of the inhibitor with 0.2 mg/ml of trypsin for 1 hour at 37°C resulted in the inactivation of antiviral activity. Except for the cell cultures treated with the inhibitor, all cultures including those exposed to the enzyme-treated inhibitor, exhibited CPE. These results strongly

suggest the protein nature of the antiviral substance produced in RA cells by reovirus type 2.

Species specificity of antiviral action. In order to demonstrate the cell species specificity of the inhibitor produced in reovirus-infected RA cells, its antiviral activity was tested in RA cells (human), BSC-1 cells (monkey origin) and L cells (mouse origin).

No inhibitory activity was observed to occur in mouse L cells even when such cultures were treated with undiluted preparations of the inhibitor. The inhibitory activity in BSC-1 cells was found to be only one-half as much as was observed in RA cells.

Mechanism of action. It has been shown through the use of metabolic antagonists that the inhibitory action of interferon is mediated by a protein whose formation requires the new synthesis of both RNA and protein in the treated cells (9,10). In order to determine whether the present interferon-like preparation also required a similar mechanism for its action, antinomycin D(AD), an inhibitor of DNA-dependent RNA synthesis (11), was used.

Actinomycin D at a final concentration of 1 μg/ml sufficient to suppress greater than 98% of cellular RNA activity, was added together with undiluted inhibitor preparations to RA cell cultures. After an exposure period of 3–5 hours, the AD-inhibitor mixture was removed, the cell cultures thoroughly washed with GKN (5×) and each tube challenged with VSV at a multiplicity of about 4. Virus yields from all cultures were determined at 24 hours postinfection by the agar plaque technique of Holland and McLaren (12).

Whereas virion production in the inhibitor-treated cultures was reduced by more than 1.0 $\log_{10}$, the virion yield from the AD-inhibitor-treated and AD-treated cultures were reduced by 0.5 $\log_{10}$. Since the yields of virions from both the AD-inhibitor-treated and AD-treated cultures were similar, the reduction in virion production was in all probability due to the antibiotic and not to residual inhibitor action. Actinomycin D at the concentration employed has been found to affect the cell's capacity to produce reovirus (13).

From the data obtained it was concluded that the mechanism of action of the inhibitor is very similar to that of interferon. Both kinds of inhibitor require the synthesis of new RNA for the production of the active protein principle which is responsible for the inhibition of virus replication.

Lack of viral specificity. Interferon has been found to be nonspecific in its inhibitory effects on a large number of RNA- and DNA-containing virions. To determine whether the present inhibitor possesses this property, its effect on the replication of a single-stranded RNA-containing virion (VSV), a double-stranded RNA-containing virion (reovirus type 2), and a DNA-containing virion (vaccinia) was investigated.

Although all three virions examined were found to be sensitive to the action of the inhibitor, they exhibited varied sensitivities. In the following order vesicular stomatitis virus, vaccinia virus, and reovirus type 2 were found to be increasingly sensitive to the inhibitor. The variation in the inhibitory effect could be attributed to the differences in sensitivity of the three virions to the inhibitor.

Discussion. The present data indicate that the inhibitory material elaborated by the stable human amnion cell lines, RA, after infection with reovirus type 2 possesses biological and physiochemical characteristics very similar to those reported for other mammalian interferons. Although the present investigation has used exclusively the human amnion cell (RA) system, preliminary studies employing other cell systems indicate that the BSC-1 cells (green monkey kidney) do not produce the inhibitor. The induction of interferon in rabbits by the complete reovirus particle and by its isolated double-stranded RNA component has recently been reported (6).

The finding that the reovirus, a double-stranded RNA-containing virion, is sensitive to the action of interferon coupled with the evidence that many single-stranded RNA and double-stranded DNA virions are also sensitive to this antiviral agent, suggests that all of these virions share a common stage(s) in their replicative sequence which is affected by interferon. Recent evidence obtained from

investigations with the Sindbis (14), Mengo (15), and vaccinia virions (16) have strongly suggested that the site of action of interferon is at the translational level of protein synthesis. Cells which have been treated with interferon are thought to produce a a protein which has been postulated to in some manner modify the host cell ribosomes so that they no longer can associate with viral messenger RNA(s). Thus, viral message is not translated and consequently, virion replication is inhibited (14–16). It would be reasonable to assume that a similar mechanism of interference can also exist in interferon-treated cells infected with reovirus. Virion specific single-stranded RNA's exhibiting complementarity in base sequence to the double-stranded viral RNA genome and associating with subribosomal and ribosomal structures have been found to appear early in the replicative cycle of the reovirus (18–20). Whether the ribosomal units in interferon-treated cells are prevented from interacting with the virion specific RNA's or not remains to be determined.

Summary. The antiviral inhibitor substance produced by reovirus type 2 in a stable human amnion cell line (RA) was found to have several properties similar to interferon. It inhibited the growth of inducing virion as well as of heterologous virions, was not sedimentable at 45,000 rpm for 1.5 hours, was not neutralized by reovirus antiserum, was not toxic to cells, was stable upon treatment at pH 2, was inactivated by trypsin, and was ineffective in cells of unrelated species (mouse). These results indicate that the reovirus can induce interferon formation in cells and, like other virions, are also susceptible to the action of the inhibitor.

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