Acad. Sci., 1962, v97, 4.

4. Foran, R. F., in Research with Primates, D. E. Pickering, ed., Tektronix Foundation, Beaverton, Oregon, 1963, p75.

5. Stern, K., Busch, S., Buznitsky, A., Am. J. Clin. Path., 1957, v27, 707.

6. Simpson, G. F., The Principles of Classification and a Classification of Mammals. Bull. Am. Museum Nat. Hist., New York, 1945, v85.

Received November 2, 1964. P.S.E.B.M., 1965, v118.

Experimental Enteric Infection of Chickens with an Avian Adenovirus (Strain 93).* (30013).

D. L. CLEMMER (Introduced by M. F. Shaffer)

Division of Epidemiology, Division of Tropical Medicine and Hygiene, School of Medicine, Tulane University, New Orleans, La.

Man is now known to be host to enteric infections with a multiplicity of viral agents. The pathogenesis of such infections, particularly at the tissue level, and the roles of humoral and cell-associated factors in the subsequent immune response are not fully understood. Although "orphan" viruses have been isolated from the gastrointestinal tract of many animal species(1), considerations of animal size and cost plus the difficulties of maintaining stock free of interfering wild viruses have rendered these systems unsuitable as experimental models for many investigators.

An earlier paper(2) described some properties of an avian adenovirus, strain 93, isolated in chick kidney tissue culture from the cloacal contents of a market chicken. The present paper reports experiments undertaken to study the behavior of strain 93 in its natural host and in particular to determine whether it might serve as a useful and convenient model for enteric viral infection with characteristics analogous to those observed in the infection of man with the human enteric viruses.

Materials and methods. Experimental animals. White Leghorn eggs were obtained from a local poultry farm and hatched in the laboratory. The chicks were fed a commercial growing mash.

Virus. Strain 93 was propagated in chick kidney tissue culture. Birds were inoculated in various experiments with 0.1 ml of undiluted tissue culture fluid containing virus at the third through eleventh passage levels. The material was introduced into the esophagus by means of a gelatin capsule.

Specimens. Throat cultures were obtained by rubbing a moistened swab over the walls of the posterior pharynx and cloacal samples by inserting a swab into the organ. Ten per cent suspensions of tissue in Hanks' basic salt solution were homogenized with a Virtis micro-assembly unit and the tissue extracts collected after low speed centrifugation. One hundredth ml-0.08 ml of bile was diluted in 3 ml of Hanks' solution. Serum for viral isolation or antibody titration was obtained from blood drawn by cardiac puncture. The processing of specimens, passage in chick kidney tissue culture for viral isolation and titration of serum antibody have been described(2).

Identification of virus isolates. All viral isolates from tissue and a sampling of those recovered from throat and cloacal cultures were typed in a standard neutralization test against 20 antibody units of anti 93 rabbit serum. Additional throat and cloacal isolates were typed by hemagglutination-inhibition (HI) against 4 units of immune chicken serum.

Results. Excretion and pathogenesis following primary oral challenge. The susceptibility of chicks to infection by the oral route was studied in 6 one-day-old chicks fed $10^{6.33}$ $TCID_{50}$ of 93 virus. Cloacal and throat swabs were taken on days 1-7, 10, 12, 14 and 17 post-inoculation. Blood for serum anti-

* This investigation was supported by a research grant from Nat. Inst. of Allergy and Infect. Dis., N.I.H., U.S.P.H.S.

body titration was obtained on the fourteenth or seventeenth post-inoculation day.

Virus was recovered from all cloacal cultures through the fourteenth day. Pharyngeal cultures were irregularly positive for 6 days and consistently positive from the seventh through the fourteenth day. All cultures were negative on day 17. With the exception of one bird, neutralizing antibody was not detectable in 1:4 serum dilution. The birds appeared normal throughout the 17-day observation period.

As the cloacal contents contain secretions from the liver, pancreas and kidneys, the contribution of these organs to the viral content of the excreta was investigated. Fourteen 3-day-old chicks were challenged with approximately $10^{7.0}$ $TCID_{50}$ of virus and 2 were autopsied on each of the following post-inoculation days: 1, 3, 5, 8, 10, 12 and 15. Pancreas, liver, kidney, bile and blood serum were examined for virus. In addition, the gastrointestinal wall from pharynx to cloaca was swabbed at 14 levels to determine the regions associated with the highest concentrations of virus. The carryover of gross amounts of lumen contents was carefully avoided.

Virus in trace amounts was recovered from: the pancreas of single birds autopsied on days 1, 3 and 5; the pancreas, kidneys and bile of 1 bird on day 8; the pancreas and bile of 1 bird on day 12. These isolations, excepting 1 each from bile and pancreas, were confirmed by reisolation from the original specimens. Liver and serum were uniformly negative for virus.

From the first through the tenth post-infection day, virus could be recovered with few exceptions from every level of the gastrointestinal tract. During this period, the highest titers were regularly found in the cecae ($10^{3.5}$-$10^{5.0}$ $TCID_{50}$/0.1 ml swab culture extract). On the third through eighth days, virus was also recovered in high titer ($10^{3.0}$-$10^{3.5}$ $TCID_{50}$/0.1 ml) from the upper and/or lower esophagus. Comparable titers were observed in the pharynx only on the eighth day. All organs appeared grossly normal.

Excretion and antibody response following primary and secondary oral challenge. Throat swabs, cloacal specimens and blood samples were obtained from 12 one-day-old chicks (Group 1) just prior to inoculation with $10^{6.50}$ $TCID_{50}$ of 93 virus. Nine uninoculated birds (Group 2) were bled at 4 days of age. Additional cultures were collected from both groups as indicated in Table I. On day 42 following primary challenge of Group 1, blood samples were taken from both groups. On day 45, Group 1 received a secondary challenge and Group 2 a primary challenge of $10^{6.50}$ $TCID_{50}$ of virus. Cultures were collected for 3 weeks thereafter and additional blood samples were drawn on days 66 and 81-82.

The recovery of virus from throat and cloacal swabs is shown in Table I. Virus in trace amounts was isolated on single occasions from the pre-challenge culture of 4 birds. These isolations could not be confirmed by reisolation from the original specimens and may represent laboratory contamination. Following primary challenge of Group 1, virus was consistently recovered from the excreta for 2 weeks with occasional isolations through day 28. Throat cultures were essentially all positive for 10 days but recovery fell markedly thereafter. Following secondary challenge, single isolations were made from the excreta of 4 birds, one on each of the first 4 post-challenge days.

Resistance to secondary challenge was not mediated by age as shown by the susceptibility of Group 2 birds to primary challenge on day 45. Among Group 2 birds excretion persisted for 11 days and no isolations were made thereafter. In addition to the earlier and abrupt cessation of cloacal excretion, primary infection in the older birds differed from that in the younger birds in the following respect: throat cultures taken from the older birds after the seventh post-challenge day generally contained virus in higher titer than the corresponding cloacal cultures as judged from the time required for the first appearance of cytopathic effect. The reverse had been observed in the younger birds.

The geometric mean titers of neutralizing antibody are shown in Table II. Sequential serum samples from individual birds were titrated in a single test. Maternally derived antibody was detectable in the pre-challenge sera of 7 of the 12 Group 1 birds. Titers

TABLE I. Recovery of Virus from Throat (T) and Cloacal (C) Cultures Following Oral Challenge of Chicks with Strain 93 Virus.

Group	Specimen	Day 0	3	7	10	14	17	21	28	35	45	46	47	48	49	52	54	56	59, 61 63, 66
1	T	2/12*	11/12	11/12	12/12	4/12	0/12	0/12	—	—	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
	C	2/12	12/12	12/12	12/12	9/12	3/12	3/12	1/12	0/12	0/12	1/12	1/12	1/12	1/12	0/12	0/12	0/12	0/12
2	T	0/9	—	—	—	—	—	—	—	—	0/9	4/9	5/9	9/9	8/9	9/9	9/9	7/9	0/9
	C	0/9	—	0/9	—	1/9	—	1/9	0/9	0/9	0/9	9/9	9/9	9/9	9/9	9/9	7/9	7/9	0/9

* No. positive/No. examined.
Group 1—primary challenge with $10^{8.50}$ $TCID_{50}$ of strain 93 on day 0.
" " secondary " " " " " " " 45.
Group 2—primary " " " " " " " 45.

TABLE II. Homologous Neutralizing Antibody Titer in Chicks Challenged with Strain 93 Virus.

Group	No. of birds	Titer on day 0–3	42	66	81–82
1	12	4.8*	313	321	241
2	9	<4*	<4	63	75

* Reciprocal of median. All other titers expressed as reciprocal of geometric mean.
Groups as in legend for Table I.

ranged from 1:4 to 1:128. The presence of maternal antibody did not influence the duration of excretion or significantly affect the antibody response observed 6 weeks after primary challenge (day 42). At this time, all birds had neutralizing antibody with titers ranging from 1:32 to 1:5780 (geometric mean 1:313). Secondary challenge produced no significant changes in titer.

Initially low levels of maternally derived antibody observed in 3 of the 9 Group 2 birds were no longer detactable on day 42. Three weeks after primary challenge (day 66), all birds had responded with antibody and titers were unchanged at 5 weeks (day 81-82). An unexplained observation is that the mean titer of Group 1 birds 6 weeks after primary challenge was significantly higher than that of Group 2 approximately 5 weeks post-challenge ($0.02<p<.05$).

Effect of prior immunization on excretion following oral challenge. Strain 93 virus in undiluted tissue culture fluid was exposed to 1:10,000 concentration of formaldehyde at 37°C for 4 days. Preliminary experiments indicated that infectious virus could no longer be recovered at this time and that the preparation was antigenic if given in sufficient dosage.

Six chickens (Group 3), 25 days of age, were bled, cultured and given a series of intramuscular injections of vaccine, 2 ml per injection, on 4 successive days. After a rest period of 3 weeks, the birds were test bled and given a booster injection of 2 ml. Ten days later (day 59) the birds were bled again and challenged with $10^{7.66}$ $TCID_{50}$ of 93 virus. A control group of 5 unvaccinated birds (Group 4) was bled at the same intervals and received the same challenge dose. Weekly pre-challenge cloacal cultures on both groups were negative.

TABLE III. Neutralizing Antibody Titers Against Strain 93 Virus Pre- and Post-Challenge.

	Day				Day post-challenge 7	15	21
Group	24	35	49	59	66	74	80
3	2.65*	4.74	14.2	227	605	1213	1021
4	3.47	2.81	2.00	2.00	3.47	83.9	145

Group 3 received 2.0 ml intramuscular formaldehyde-inactivated virus on days 25-28 and 49.
Group 4 unvaccinated control.
Oral challenge on day 59 with $10^{7.66}$ $TCID_{50}$ of strain 93.
* Reciprocal of geometric mean. Titers <1:4 assigned the value of 1:2.

The results of neutralizing antibody titrations are presented in Table III. On day 29 maternal antibody in low titer was present in the serum of one bird in each group. Antibody response to the vaccine was observed in half the Group 3 birds 3 weeks after the fourth injection. Ten days after the booster dose, titers ranged from 1:64-1:1446 (geometric mean 1:227). At this time no antibody was detectable in the sera of Group 4 birds.

Following challenge on day 59, all of the Group 3 birds experienced a 4-fold or greater rise in titer, except the bird with the highest pre-challenge titer, 1:1446. The major antibody response among Group 4 birds occurred during the second post-challenge week.

The effect of vaccine-induced antibody on excretion can be seen in Table IV. The mean virus recovery rate from throat cultures for Group 3 was 27.8% compared to 73.4% for Group 4. This difference in proportions is significant at the $p<.001$ level. The proportion of positive cloacal cultures in the two groups was not significantly different. Judging from the time at which cytopathic effect was first observed, specimens from the immunized birds after the second post-challenge day usually contained less virus than those from the control group. The short duration of cloacal excretion in Group 4 and the prominence of throat excretion in the latter phase of infection are consistent with the observations following primary challenge of older birds shown in Table I.

Correlation of neutralizing and HI antibody response. Sequential sets of sera, 4 to 8 sera per set, drawn at 6-10-day intervals from 10 birds experimentally infected with 93 virus were titrated for neutralizing and HI antibodies. In each case, the first appearance of HI antibody coincided with or followed by one serum sample the first appearance of neutralizing antibody. The titers were significantly correlated ($r = 0.59$, $p = 0.01$) but the correlation was not of such a high order that the HI titer could be used with certainty to predict the neutralization titer. This is illustrated in a scattergram (Fig. 1). HI antibody was usually detectable in sera with neutralizing titers of >1:32 but in 10 sera with neutralizing titers of $\geq$ 1:1024, the HI titers ranged from 1:20 to a maximum of 1:320. The persistence of the 2 types of antibody is not known.

Discussion. Infection following oral inoculation of chicks with strain 93 virus is primarily confined to the gastrointestinal tract with occasional extensions by an unknown route to the pancreas and, rarely, to the kidneys and gall bladder. Certain similarities to human enterovirus infection are evident: prompt excretion of virus in the feces; recovery of virus from the pharynx; development of serum antibody measurable by neutralization and HI techniques; resistance to secondary challenge independent of the neu-

TABLE IV. Recovery of Virus from Throat (T) and Cloacal (C) Cultures Following Oral Challenge with Strain 93 Virus.

Group	Specimen	Day post-challenge 2	4	7	9	11	14	Total	Mean virus recovery rate (%)
3	T	0/6*	2/6	1/6	4/6	2/6	1/6	10/36	27.8
	C	6/6	6/6	6/6	3/6	0/6	0/6	21/36	59.3
4	T	1/5	5/5	5/5	5/5	5/5	1/5	22/30	73.4
	C	5/5	5/5	5/5	4/5	1/5	0/5	20/30	66.6

Groups as described in legend for Table III.
* No. positive/No. examined.

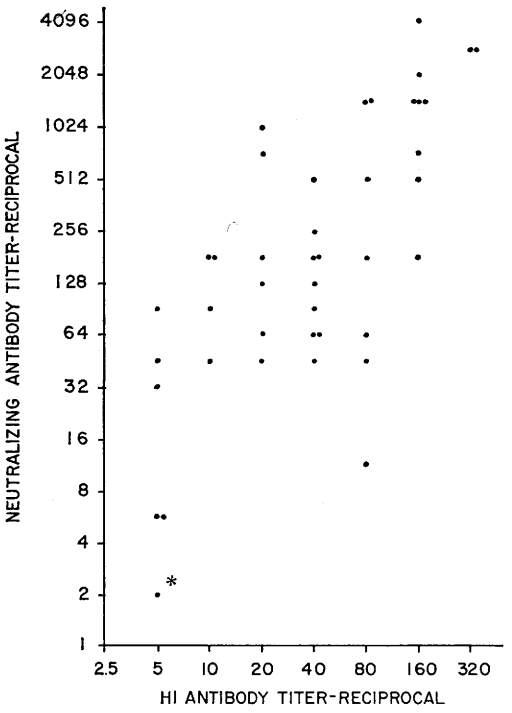

FIG. 1. Correlation of neutralizing and hemagglutination-inhibiting (HI) titers *vs* strain 93 virus. Neutralizing antibody titers of <1:10 assigned a value of 1:5. Hemagglutination-inhibiting titers of <1:4 assigned a value of 1:2.

* 33 observations.

tralizing antibody titer and lack of age-associated resistance to primary infection. As is commonly the case with human enterovirus infection, overt signs of disease were absent. The limited effect of moderate levels of neutralizing antibody, maternal or vaccine-evoked in origin, on susceptibility to primary enteric infection and the greater effect on excretion of pharyngeal virus has been observed in experimental poliovirus infections of both humans(3) and animals(4). Similar effects were observed in the present study.

The delayed antibody response in birds challenged at one day of age may be comparable to that observed by Pagano *et al*(5) in infants less than 35 days old at the time of oral vaccination with attenuated poliovirus. Primary response in older birds, as has usually been observed in older human vaccinees, occurred promptly.

It is of interest that strain 93 is an adenovirus rather than a true enterovirus. The enteric nature of certain human adenovirus infections is now recognized but extensive experimental studies such as those with the attenuated poliovirus strains are lacking. The available data however suggest that the behavior of certain members of the 2 groups of agents in the human host is not dissimilar(6, 7). Studies of the present model may yield data useful in understanding the dynamics of both types of human enteric virus infections. Current investigations of avian enteric infection with strain 93 virus are being conducted at the tissue and cellular level.

Summary. The behavior of strain 93, an avian adenovirus, was studied in its natural host following oral inoculation. Infection was primarily confined to the gastrointestinal tract with the highest concentrations of virus occurring in the esophagus, the terminal ileum and the cecae. Following oral challenge of day-old chicks, cloacal excretion persisted for approximately 14 days and pharyngeal excretion for 10-14 days. Neutralizing antibody response was detectable at 6 weeks but not at 2 weeks post-challenge. Birds after recovery were refractory to secondary challenge regardless of antibody titer. Cloacal and pharyngeal excretion following primary challenge of 6- or 8-week-old birds persisted for 9-11 days. Neutralizing antibody response occurred during the second post-challenge week. Moderate levels of antibody induced by killed virus vaccine inhibited recovery of virus from the pharynx following primary challenge but had no effect on the duration of cloacal excretion. The development of hemagglutination-inhibiting antibody paralleled that of neutralizing antibody but at lower titers.

The counsel of Dr. Stephen P. H. Mandel in statistical analysis of the data is gratefully acknowledged. Miss Marie Belle Cameron and Mrs. Carol Malfer afforded excellent technical assistance.

1. Kalter, S., Bull. WHO, 1960, v22, 319.
2. Clemmer, D. I., J. Infect. Dis., 1964, v15, 386.
3. Dick, G. W. A., Dane, D. S., McAlister, J., Briggs, M., Nelson, R., Field, C. M. B., Brit. Med. J., 1961, v2, 266.
4. Bodian, D., Nathanson, N., Bull. Hopkins Hosp., 1960, v107, 143.
5. Pagano, J. S., Plotkin, S. A., Koprowski, H., New Eng. J. Med., 1961, v264, 155.

6. Rosen, L., Hovis, J. F., Ball, J. A., Proc. Soc. Exp. Biol. and Med., 1962, v111, 166.
7. Kasel, J. A., Evans, H. E., Spickard, A., Knight, V., Am. J. Hyg., 1963, v77, 265.

Received November 4, 1964. P.S.E.B.M., 1965, v118.

Time Study of Incorporation *in vivo* of Inorganic Phosphate (P^{32}) into Phospholipids of Mitochondria.* (30014)

JAMES E. MILLER AND W. E. CORNATZER
Guy and Bertha Ireland Research Laboratory, Department of Bochemistry, University of North Dakota, School of Medicine, Grand Forks

Phospholipids are found in the membranes (1), mitochondria, nuclei, and microsomes (2,3) of the cell. Paper chromatographic techniques are capable of providing composition of various phospholipids from tissues (4,5), and subcellular fraction(6,7) of the cell. The incorporation *in vivo* of inorganic phosphate (P_i^{32}) into the individual phospholipids of rat tissues has been reported(8, 9). However, an *in vivo* time study on uptake of P_i^{32} into individual phospholipids of liver mitochondria has not been reported. This study reports the data on a time study of the incorporation of P_i^{32} into mitochondrial phospholipids of rat liver and hamster liver and kidney.

Methods. Male albino rats of the Sprague-Dawley strain, previously maintained on Purina Laboratory chow, weighing 120-150 g, were used in the time uptake study. Male hamsters, weighing 120-160 g were maintained on Purina Laboratory chow pellets. The animals were injected with 200 μc of P_i^{32} and sacrificed at 0.5, 1, 2, 4, 6, 8, and 10 hours later by decapitation. The tissues were quickly removed, rinsed with cold water and placed on cracked ice. The organs were immediately weighed and homogenized in cold 0.25 M sucrose in a Potter-Elvehjem homogenizer with teflon pestle. The kidney was diluted to 25 ml with the cold sucrose solution and the liver to a concentration of 1 g per 8 ml. Aliquots of 5 ml were pipetted into 10 ml of a cold solution of 10% trichloroacetic acid plus 0.4 M $MgCl_2 \cdot 6H_2O$(10) and kept 3°C overnight and inorganic phosphorus(11) and radioactivity(12) analysis performed on the supernatant. The remaining homogenates were centrifuged(13) in the cold for 15 minutes at 800 $\times$ *g* to remove the nuclei. The nuclear pellet was rehomogenized in sucrose and centrifuged and the supernatants combined. Mitochondria were separated from the combined supernatants by centrifugation at 10,000 $\times$ *g* for 15 minutes. The mitochondrial pellet was twice resuspended in sucrose and centrifuged to wash out contaminating microsomes. This procedure removed over 99% of the microsomes as determined by assaying for microsomal glucose-6-phosphatase(14).

The lipids were extracted from the mitochondria by extracting once with 8 ml of methanol and twice with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at room temperature. This was followed by 4 extractions with 8 ml portions of $CHCl_3$-CH_3OH (2:1) at 55°C. Each extraction was for 15 minutes in a shaking water bath followed by centrifugation for 3 minutes at 1,600 $\times$ *g* to facilitate removal of the solvent. The organic solvents of the pooled extracts were evaporated on a Rinco Rotating Vacuum Evaporator (Rinco Instrument Co.) at 35° and the water removed by lyophilization in a Freeze-Dryer (Virtis Co., Inc.). The lipids were taken up in chloroform and the phospholipids separated by chromatography on silicic acid impregnated glass filter paper(15). The solvent system was diisobutyl ketone:acetic acid: water:benzene (160:50:8:7, v/v). The chromatograms were dried, stained with Rhodamine 6G and the phospholipids identified with ultraviolet light(16). The individual phosphatides were extracted with 1 N metha-

* Supported by Contract AT 11-1-479 from U. S. Atomic Energy Comm. and U.S.P.H.S. Grant.