phosphorus seems to invalidate use of the fall in serum concentration of this ion as an index of peripheral utilization of carbohydrate.

Summary. The normal rabbit, treated with hypoglycemic doses of phenethylbiguanide, shows a significant increase in serum inorganic phosphate levels. In this respect the action of phenethylbiguanide differs from that of insulin and sulfonylurea oral hypoglycemic agents, and closely resembles that of structurally related diguanidine, Synthalin A. Increase in serum inorganic phosphorus levels is consistent with inhibition of oxidative enzyme systems of the citric acid cycle in presence of phenethylbiguanide, which suggests that inorganic phosphorus might escape into the blood stream due to decreased binding of high energy phosphorus.

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Further Studies on 2060 Virus.* (25261)

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Previous reports have described isolation of an apparently new viral agent, designated 2060, and have presented the case for etiological relationship to mild, afebrile, coryzal illnesses(1-4). Antigenic relationship, but not identity to the JH virus was also noted. The latter agent was isolated independently by Price and was also thought to be the cause of similar illnesses in man(5-7). Both of these viruses appear to have characteristics in common, including rate of growth and metabolic requirements, stability, cytopathogenic effects and infectivity for the same hosts. Certain of these features of 2060 are described herein.

Methods. Cultures (TC) were prepared with 0.25% trypsin (Difco, 1-250) dispersed kidney cells from young adult male Rhesus monkeys, planted at a 1-400 dilution of cells packed at 800 rpm for 10 minutes and grown at 35°C in stationary tubes and prescription bottles with 0.5% lactalbumin hydrolysate (Nutritional Biochemicals) and 2% calf serum (Microbiological Associates) in Earle's solution. After 4 to 7 days they were washed twice and maintained with Mixture 199 or Eagle's medium (Microbiological Associates), or the 2 combined in equal parts, with care to

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lower pH with CO_2 sufficiently so that it would remain within 7.0-7.2 at 35°C. Cell lines derived from human amnion and lung and kitten lung (obtained from Dr. C. Pomerat) and human kidney (Chang) were propagated in 199 or Eagle's with 10% horse serum and maintained for experiments as above. Stock virus, 2060 TC_{5-7}, was prepared in tube and bottle cultures inoculated with 0.1 ml/ml of TC fluid containing 10^{3-4} $TCID_{50}$/0.1 ml, harvested after 5 to 7 days growth at 35°C, clarified by centrifugation at 1500 rpm for 10 minutes, quick frozen and stored at −65° C. Infectivity ($TCID_{50}$) was determined by inoculating 0.1 ml of 10-fold dilutions of TC fluids into groups of 4 monkey kidney cultures. Readings were made at intervals up to 7 days and a culture was considered infected by appearance of characteristic cytopathology. Neutralization titers were determined by adding 10^3TCID_{50} of virus to equal quantities of 2 fold dilutions of inactivated sera (56°C, 30 minutes), incubating at 4°C for 30 minutes, and inoculating groups of 3-4 cultures with 0.1 ml of serum-virus admixture. Titers were calculated on the basis of initial dilution of serum inhibiting the development of cytopathogenic effects at 7 days.

Results. Growth in stationary cultures. To correlate yield of infectious virus with development of cytopathogenic changes in stationary cultures, a small infecting dose was used as shown in Table I. Increments in infectious virus and proportion of involved cells reached a plateau despite the presence of many unaffected cells. By replacement of maintenance medium virus titers could be sustained for several additional days until a majority of the cells had undergone complete disruption. In other experiments established cell lines, including human kidney, human amnion and lung and kitten lung, demonstrated no cytopathogenic effects and produced no infectious virus after inoculation of 2060.

TABLE I. Growth of 2060 TC_6 in Stationary MK Cultures.*

Days after infection	Degree of CP effect	Virus yield, ID_{50}/0.1 ml (log)
1	0	1.5
2	+	2.0
3	++	3.0
5	++ ++	3.5
7	++ ++	3.7

* Inoculum was 10^2TCID_{50}. Groups of 4 cultures were harvested at intervals shown.

TABLE II. Effect of Various Constituents of Eagle's Medium on 2060 Virus Yield in Monkey Kidney Cultures.

Maintenance media*	Virus yield, ID_{50}/0.1 ml (log)	
	A	B
V + G + AA	4.0	3.0
V + G	3.8	
V + AA	3.2	2.0
G + AA	3.0	

* Cultures maintained 3 days in modified media prior to inoculation and same medium replaced at time of infection.

V, vitamin mixture, 1%; AA, amino acid mixture, 1%; G, glutamine, 200 mM, 1%.

Nutritional factors. Since virus yield and cytopathogenic effects were greater in monkey kidney cultures maintained with synthetic media than those containing animal sera or embryo extract, studies were designed to evaluate the contribution of constituents(3). Experiments comparing Eagle's with Mixture 199 had revealed a slightly greater virus yield and a fairly marked enhancement of cytopathogenic changes with the former. Accordingly, the relative effects of glutamine, vitamins and amino acids in Eagle's medium were determined by selective omission of each (Table II). Although no single critical factor was demonstrated, absence of glutamine or vitamins caused more reduction in virus yield.

Effect of pH on growth and stability. It had previously been noted that fall of pH in culture fluids (<6.8) resulted in reduction in viral multiplication and cytopathogenic effects. Adjustment of pH with $NaHCO_3$ solution to 7.6 or above also was followed by lessening of cytopathogenic effects and virus yield remained low. Experiments comparing pH 7.0-7.2 with 7.6-7.8 revealed a 10-100 fold reduction in virus yield and sensitivity to infection at the higher pH. This was observed with either Eagle's or Mixture 199 as maintenance medium. The stability of infectious 2060 in solutions with pH ranging from 1.0 to 11.4 was determined at 4° and

TABLE III. Stability of Infectious 2060 Virus in Different Hydrogen Ion Concentrations and Varying Times and Temperatures of Exposure.

	Conditions and resulting infectivity titers*			
	4°C		35°C	
pH	30 min.	24 hr	30 min.	24 hr
	ID$_{50}$†			
1.0	.0	1.0	.0	.0
5.0	2.5	1.0	2.5	.0
7.1	2.5	—	2.5	1.5
8.8	1.5	1.0	.0	.0
11.4	.0	.0	.0	.0

* Untreated control at pH 7.0 was $10^{2.8}$TCID$_{50}$.
† Reciprocal of logarithm of infectivity titer/0.1 ml.

35°C as shown in Table III. In these experiments the pH of Mixture 199 was adjusted with 1N HCl or NaOH and aliquots sealed in glass ampoules immediately after addition of virus. Following incubation of similarly prepared aliquots for 30 minutes and 24 hours respectively, the pH was adjusted to neutrality and infectivity titers determined. The greatest degree of stability began at neutrality and extended to the acid side. In contrast, even a half hour at 4°C in an alkaline solution caused loss of infectious virus. Increased temperature or time regardless of pH was accompanied by reduction in infectivity titers. Accordingly, 2060 could not be called a very stable virus, and the preference for the acid side of neutrality for maintenance of infectivity demonstrated the need for care in control of hydrogen ion concentration.

Thermal stability. The effect of temperatures of 35-56°C on infectivity was determined at pH 7.2. Aliquots of virus were sealed in glass ampoules and heated for variable periods at the temperatures shown in Fig. 1. Inactivation rate was quite rapid at 56°C for 10 minutes and decreased progressively at lower temperatures. The reduction that occurred at 35°C was appreciable and undoubtedly had a bearing on yield of infectious virus especially when a prolonged growth period was employed. These results classify 2060 as relatively unstable in regards to increased temperature. Methods were not available to determine the stability of noninfectious antigen.

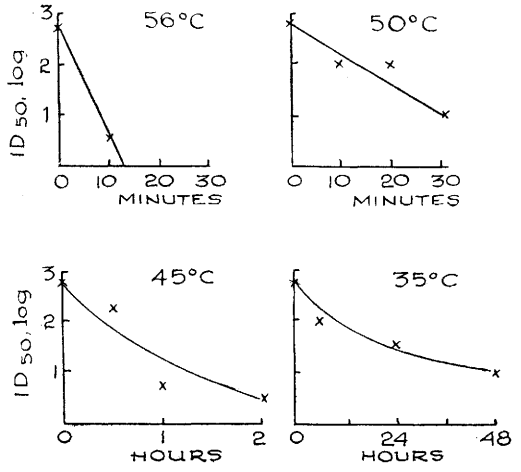

FIG. 1. Rates of reduction of infectivity titers of 2060 by temperatures of 35-56°C.

TABLE IV. Antigenic Relationship between 2060 and JH Viruses by Reciprocal Neutralization with Animal and Human Antisera.

	Neutralization titers‡			
	2060		JH	
Antisera	Acute	Conv.	Acute	Conv.
Animal*				
2060, monkey	11	91	<8	8
		128		32
JH, rabbit	<4	<4	4	"
2060, "	"	128	<4	<8
Human†				
DEF	16	128	8	11
JAB	32	16	8	45
BB	16	128	11	64
WH	64	45	"	11

* Prepared by intrav. inoc. of 10 ml of virus with a titer of $10^{8.5}$TCID$_{50}$/0.1 ml and bled at 14 days. A second inj. was given to the monkey at 14 days and another convalescent blood was obtained at 28 days.
† Representative serum pairs from cases of respiratory disease demonstrating the various types of responses to each virus.
‡ Reciprocal titers of initial dilutions of sera inhibiting $10^{2.5}$TCID$_{50}$.

Antigenic relation to JH virus. Neutralization titers of animal and human antisera were determined simultaneously with 2060 and JH viruses (Table IV) in order to evaluate their degree of antigenic differentiation. Considerable specificity was observed with antisera from animals immunized by a single inoculation of virus, while a second injection increased the neutralization titer to the heterologous virus. Closer relationship was demonstrable with human sera. Four fold or greater increments in neutralization titers with both viruses were found in 32 of 57 human serum

pairs. Increases in neutralization titers to only one virus was observed in the remainder.

Prevalence of infection as determined by neutralization titers of human sera. Acute and convalescent sera were collected from young adults with mild, afebrile respiratory illnesses with coryza as the predominant symptom, although mild sore throat and nonproductive cough were also present in some. Group A streptococci were not found by throat cultures and there was no antibody increase by hemagglutination-inhibition with recent influenza A and B strains, except in those recently vaccinated, or by complement-fixation with adenovirus in these individuals. Four fold or greater increments in neutralization titers were observed in 6 of 13 serum pairs obtained during the spring of 1957 and in 10 of 31 obtained during the fall of 1957 in the New Orleans area. Inoculation of garglings from the above persons into monkey kidney cultures in several instances produced cytopathogenic changes characteristic of 2060, but the agents were repeatedly lost on subsequent passage before identification could be accomplished.

Although neutralization titer increments were readily reproducible in separate experiments, titers of individual sera varied considerably. Factors such as degree of development of cytopathogenic changes and effective quantity of infectious virus had a bearing on these results. The significance of neutralization titers in the absence of increases between serum pairs determined simultaneously was, accordingly, subject to question.

Discussion. Although preliminary studies had indicated that 2060 was a very stable agent with relatively slow rate of growth, more detailed observations have shown a considerable degree of instability of the infectious particle in regards to pH and temperature extremes. This demonstrates the need for determinations of characteristics of a virus that include several dimensions simultaneously, including time as a variable rather than a constant. Even rate of growth in tissue culture may be dependent on these properties as well as the type of cell and the constituents of the culture fluid. Serum, later found to be inhibitory to rate of viral multiplication, had previously been included in maintenance media(2,3). On the other hand, the use of roller tubes had produced a greater yield of infectious virus than that derived from stationary cultures. This has been observed by others (8). Such factors influenced results of neutralization titrations and may serve to explain lack of reproducibility of titers in separate experiments. Although titer increments were accurately repeatable, measurements of antigenic differences between JH and 2060 might have been overly sensitive, since lesser amounts of antibody were needed to inhibit smaller quantities of operationally infective virus. Similar considerations reduced the significance of titers of neutralization determined on single human sera and made surveys of antibody levels in various populations of doubtful merit.

Summary. In monkey kidney cultures increments in infectious 2060 could be correlated with the degree of observed cytopathology. The absence of glutamine or vitamins contained in Eagle's medium had some effect in lessening yield of infectious virus, but alterations in pH away from neutrality were more significant in reducing viral multiplication. Infectious 2060 was relatively unstable as evidenced by the inactivation rate at temperatures of 35°C and above and pH below 5.0 and above 7.1. JH virus and 2060 were shown to be related antigenically, but were not identical, as shown by reciprocal neutralization titrations with animal and human antisera. Additional instances of neutralization titer increments in man following symptoms of mild respiratory illness have been presented.

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