a single washing with normal, heat-inactivated, absorbed rabbit serum diluted 1:100 in Sorenson phosphate buffer. The washed erythrocytes were resuspended in rabbit serum similarly diluted with Sorenson phosphate buffer.

Erythrocytes exposed to antigen without pretreatment with bis-diazotized benzidine did not show hemagglutination in the presence of antibody, presumably because of failure of the cells to bind the antigen. Erythrocytes treated with bis-diazotized benzidine, but not coupled with antigen, also showed no hemagglutination. When gastrins I and II were used as the detector antigen, complete antibody titers of 256, 128, and 512, respectively, were determined in three rabbits 6 weeks after initial immunization. Comparable titers were determined by use of stage-I gastrin, gastrin pentapeptide, or gastrin tetrapeptide as detector antigen coupled to the erythrocytes. The use of goat anti-rabbit gamma globulin in the detection procedure increased the titers mentioned to 1024, 512, and 2048, respectively.

Since a pure antigen was not used to incite antibody production, it was necessary to establish the specificity of the antibody by several different methods. This specificity was first demonstrated by hemagglutination with gastrin pentapeptide, gastrin tetrapeptide, and gastrins I and II, as well as stage-I gastrin, as detector antigens on the erythrocytes. For hemagglutination to occur with the peptides as detector antigens, specificity must necessarily be directed to this portion of the gastrin molecule.

Specificity of the antibody was also demonstrated by inhibition of passive hemagglutination (*7*); a titer of antiserum to gastrin gave 4+ hemagglutination. When gastrins I and II were used as detector antigen coupled to the erythrocytes, hemagglutination was completely inhibited when less than 0.25 μg of stage-I gastrin, gastrins I and II, gastrin pentapeptide, or gastrin tetrapeptide was preincubated with 0.1 ml of the antiserum (Table 1). The relative effectiveness of gastrins I and II, pentapeptide, and tetrapeptide as inhibitors of hemagglutination appears to be related to their molecular weights. The lesser amounts of the two peptides (that of gastrins I and II) necessary for complete inhibition of hemagglutination is noted as evidence of the specificity of the antibody for a specific portion of the gastrin molecule.

One cannot compare the relative effectiveness of stage-I gastrin as an inhibitor on a molar basis because of the impurities contained in the preparation. The increased amounts necessary for complete inhibition of hemagglutination may reflect the impurities contained in the stage-I gastrin preparation. This idea is supported by the data obtained when stage-I gastrin was used on the erythrocytes as the detector: in this case smaller amounts of stage-I gastrin than of gastrins I and II were required to inhibit hemagglutination completely (Table 1).

Table 1. Specificity of antibody as determined by inhibition of passive hemagglutination. The inhibitor was preincubated with 0.1 ml of antiserum before reaction in the passive hemagglutination system. Detector was the antigen coupled to the erythrocytes by bis-diazotized benzidine. Complete inhibition is expressed as the least amount of inhibitor necessary to inhibit hemagglutination completely. Each value is the mean of six determinations.

Inhibitor	Complete inhibition (μg)
Detector: gastrins I and II	
Stage-I gastrin	0.25
Gastrins I and II	.16
Gastrin pentapeptide	.10
Gastrin tetrapeptide	.05
Detector: stage-I gastrin	
Stage-I gastrin	0.12
Gastrins I and II	.33
Detector: gastrin pentapeptide	
Stage-I gastrin	0.08
Gastrins I and II	.10
Gastrin pentapeptide	.14
Detector: gastrin tetrapeptide	
Stage-I gastrin	0.11
Gastrins I and II	.12
Gastrin tetrapeptide	.20

Specificity of the antibody was further determined by experiments in which antibody activity was completely absorbed by erythrocytes coupled with gastrins I and II, gastrin pentapeptide, or gastrin tetrapeptide. The absorption was complete when stage-I gastrin, gastrins I and II, or gastrin pentapeptide was used as the detector antigen coupled to the erythrocytes.

Each of these three methods served to establish that the specificity of the antibody is directed not only toward the original antigen (stage-I gastrin), but also to the pure gastrins I and II. In addition, specificity of the antibody is directed toward a portion of the gastrin molecule: that is, the gastrin pentapeptide and the gastrin tetrapeptide.

In fractionation of the antiserum on a column of Sephadex G-200, the antibody was eluted in the fractions corresponding to a 19*S* immunoglobulin (*10*).

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Viral Hemorrhagic Encephalopathy of Rats

Abstract. *A virus has been isolated and serially passed in suckling rats; it causes an acute fatal paralysis associated with hemorrhage and necrosis in the brain and spinal cord. The agent is relatively resistant to heat and ether, is about 20 millimicrons in diameter, and is antigenically closely related to rat virus. Its isolation resulted from the study of occasional cases of paralysis in adult rats after administration of cyclophosphamide.*

In order to produce transient immunosuppression, 500 adult Lewis rats were given cyclophosphamide in a single intraperitoneal dose of 100 to 150 mg per kilogram body weight. Paralysis, usually of the hind limbs, and usually beginning 2 to 3 weeks after cyclophosphamide injection, was observed in less than 2 percent of treated animals, but never in untreated animals.

Four affected rats were killed, under pentobarbital anesthesia, by perfusion with sterile balanced saline, within 1 to 3 days after onset of paralysis. The brain and cord were combined and homogenized in balanced saline containing

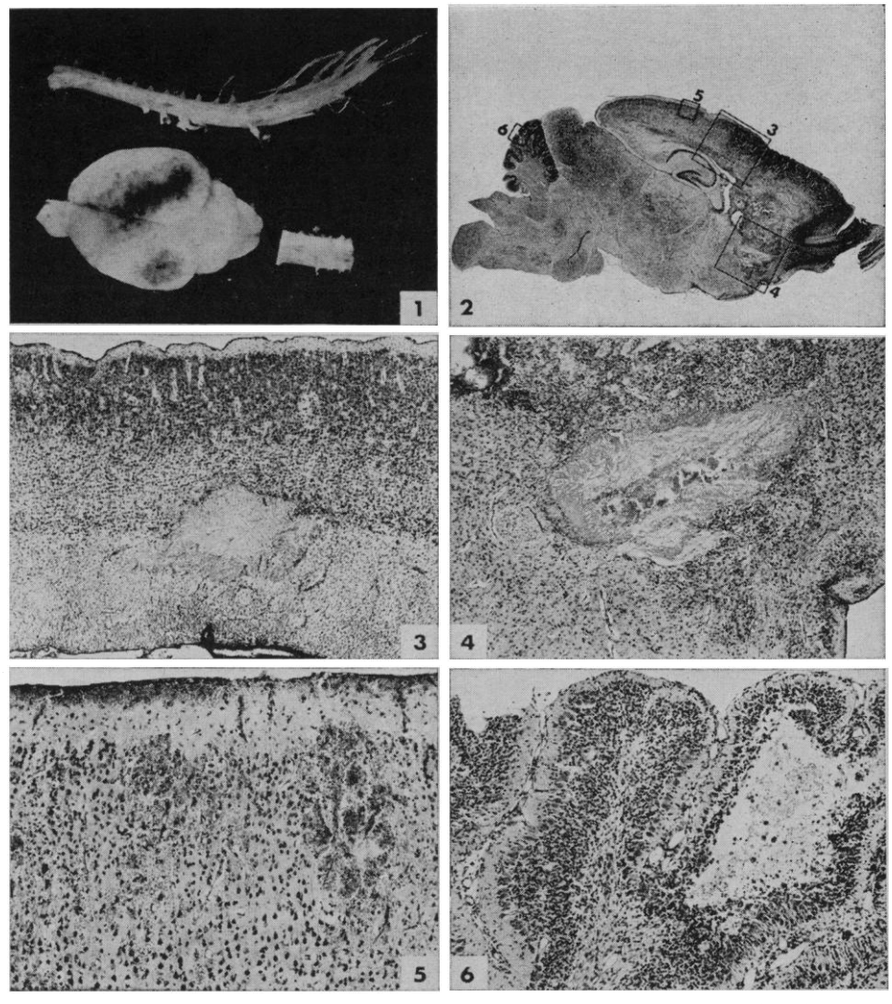
0.75 percent bovine plasma albumin; the homogenate was centrifuged at 2500 rev/min for 20 minutes at 4°C, and the supernatant was inoculated into 1- to 3-day-old suckling Wistar rats. Two of the four homogenates produced acute paralysis in suckling rats, by either intracerebral or intraperitoneal routes of inoculation, with onset usually 7 days after injection; most animals died within 3 days of onset, while some survived as long as 2 weeks with marked stunting of growth and signs of central nervous system (CNS) dysfunction.

Evidence that an infectious agent has been isolated is provided by serial passage through suckling rat brains; at present the agent is in the fourth passage with a cumulative dilution of 10^{-18}. With age, rats rapidly become resistant to this agent. Thus, 2-week-old sucklings were unaffected by inoculation of a 1:10 dilution of a pooled virus (HER1 SR3) which, when injected intracerebrally, has a titer of 10^7 LD_{50} (lethal dose, 50 percent effective) per 0.02 ml in 1- to 2-day-old animals. Mice (ICR strain) are much less susceptible than rats, since inoculation of a 1:10 dilution of pool HER1 SR3 produces fatal paralysis in about 50 percent of 1- to 2-day-old sucklings by the intracerebral route and no illness by the intraperitoneal route. The HER (hemorrhagic encephalopathy of rats) agent produces a marked cytopathic effect within 4 to 5 days of inoculation of large doses into primary rat embryo tissue culture.

Suckling rats killed by perfusion in the terminal stage of infection show dramatic gross hemorrhages in the substance of the brain (Fig. 1). Microscopically, two types of focal lesions are seen: (i) hemorrhage and (ii) liquefying necrosis, with relatively few inflammatory cells, except at the margins of necrotic areas (Figs. 2–6). The two types of lesions may occur separately or together, and they are particularly common in the cerebral cortex, corpus callosum, and white matter of the cerebellum. Adult rats, developing paralysis after administration of cyclophosphamide, had less severe CNS lesions. There were small, scattered focal hemorrhages in the spinal cord, more commonly in white than gray matter, and occasional foci of apparent demyelination without overt necrosis. In other tissues of both adult and suckling rats no obvious hemorrhage or necrosis has been observed, but in the adults that received cyclophosphamide mesenteric lymph nodes show erythrophagocytosis and hypoplasia of lymphoid elements.

The hemorrhagic agent has been passed through Selas 03 (100 mμ) and Millipore 50-mμ filters. In order to rule out a nonfilterable organism producing a filterable toxin, the agent has been propagated serially with filtration between each of five passages, and it is fully active after a cumulative dilution of 10^{-10} after the first filtration. Treatment with 20 percent diethyl ether at 4°C for 18 hours (*1*) reduces the titer of pool HER1 SR3 by less than tenfold. Heating ot 50°C for 1 hour reduces the titer by less than 100-fold (*2*).

Clarified homogenates of brains of moribund suckling rats, identical to those used for virus passage, were examined for virus particles by electron microscopy. Suspensions were stored at −65°C until needed, thawed, treated briefly with high-frequency sound, shaken for 5 minutes in diethyl ether, filtered through Celite, layered on a cesium chloride density gradient, and then centrifuged for 18 hours at 25,000 rev/min (*3*). The resulting gradient was fractionated by collecting drops coming through a pinhole in the bottom of the tube. Each fraction was checked for density, and prepared for electron microscopy (*3*). Most of the cell debris banded in regions having densities less than 1.34 g/cm^3. Numerous particles of the type shown in Fig. 7 were observed in fractions having a density of 1.37 to



Figs. 1–6. Tissue from a suckling Wistar rat, inoculated intracerebrally with the HER agent. Paralyzed and perfused 7 days after inoculation. Fig. 1. Brain and spinal cord, showing gross hemorrhages throughout central nervous system (about 1.25 ×). Fig. 2. Brain, parasagittal section, gallocyanin stain (about 2.5 ×). Location of areas shown in Figs. 3 to 6 are indicated by boxes. Fig. 3. Cerebral cortex and corpus callosum. An area of sharply defined necrosis extends from corpus callosum into subcortical gray matter (about 20 ×). Fig. 4. Basal ganglia. An area of necrosis with central hemorrhage has destroyed part of caudate-putamen nucleus (about 25 ×). Fig. 5. Cerebral cortex. Molecular layer is partly replaced by subpial hemorrhage, and two foci of hemorrhage are seen in deeper layers of cortex (about 50 ×). Fig. 6. Cerebellum. Complete focal destruction of white matter of one folium of cerebellum (about 50 ×).

Table 1. Antigenic relationship of HER virus and rat virus in hemagglutination-inhibition (HI) tests.

Serum from rabbit immunized with	Titer against 6 HA units of	
	R1SR3 TC1	3PRE 308
HER agent, R1SR3	160	160
Rat virus, 3 PRE 308	80	160
Rat virus, H-1	<20	<20
Normal rat brain	<20	<20
Before immunization	<20	<20

1.39 g/cm^3. These particles were about 20 mμ in diameter, somewhat angular in outline, and occasionally appeared to have knob-like subunits on their surface (see arrow in Fig. 7). No other virus-like particles were seen in these preparations. Assays of dialyzed fractions in suckling rats revealed that most of the infectivity was associated with the fractions containing most of the particles. These results are consistent with the conclusion that the biological activity (production of hemorrhagic encephalopathy) of these suspensions is associated with a small dense virus physically similar to those previously described (*4*).

Limited trials have shown that the HER agent (infected rat brain or rat embryo tissue culture fluid) agglutinates red blood cells from guinea pigs but not those from chicken, sheep, rhesus monkey, or man (type O). Our first tests indicate that serum from rabbits immunized with HER virus will inhibit hemagglutination. Furthermore, hemagglutination-inhibition tests (Table 1) with rat virus antigen and antiserum (provided by L. Kilham) indicate a definite antigenic relationship to strain 3 PRE 308 but not to the strain H-1 of rat virus (*4*).

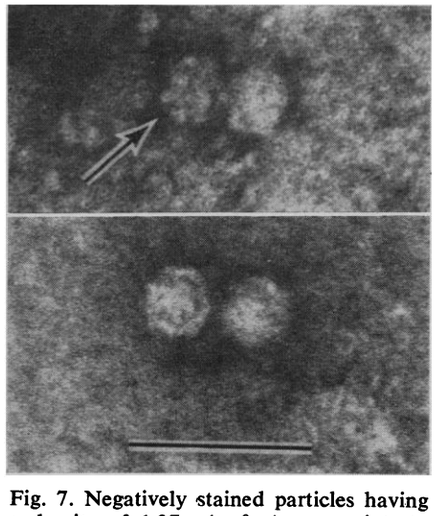


Fig. 7. Negatively stained particles having a density of 1.37 g/cm^3. Arrow points to knob-like subunits on the surface of one particle (bar = 50 mμ).

Hemorrhagic encephalitis in infected rats has been seen on rare occasions by Kilham and Margolis (*5*) during their extensive work with rat virus (*4*), although we have not found any other reports of a virus which attacks the central nervous system in the manner described above (*6*). Together with our experimental results this suggests that we have isolated a strain of rat virus with biological properties quite different from those of strains previously isolated. We propose HER virus as a tentative name for our isolates and suggest that they be called the HER strains when they can be assigned a definitive taxonomic classification.

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Suppressor Selection for Amino Acid Replacements Expected on the Basis of the Genetic Code

Abstract. *In studies with A protein mutants of the tryptophan synthetase of* Escherichia coli, *missense supressors have been used to select for codon changes and corresponding amino acid replacements that are normally unobservable. The technique has permitted the detection of additional amino acid replacements expected on the basis of the genetic code.*

Extensive genetic and biochemical studies with the A-gene–A-protein system (tryptophan synthetase) of *Escherichia coli* K-12 have resulted in the identification of many single amino acid substitutions associated with single mutational events (*1, 2*). By examining the amino acid changes associated with mutations from prototroph to auxotroph to prototroph, multiple amino acid substitutions at the same position in the A protein have been detected. In all but one case, the codons for the particular amino acids in each substitution are related in that a single nucleotide change in an RNA codon can account for the change. For example, mutant A46, a strain in which there is an amino acid change from glycine to glutamic acid at position 210 in the A protein, yields revertants containing either glycine, alanine, or valine at this position (Fig. 1). From the RNA codons shown in Fig. 2 (*3*) it is apparent that single nucleotide changes in the A46 glutamic acid codon, which is probably GAA (*4*), should also result in the replacement of glutamic acid by aspartic acid, lysine, and glutamine (*1, 2*). The failure to find these substitutions in the A protein of the revert-

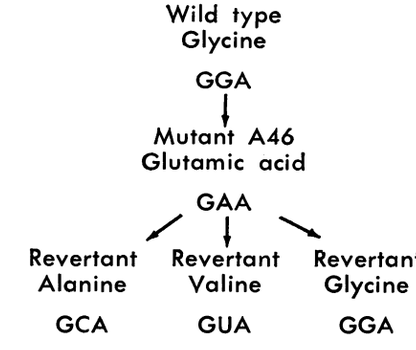

Fig. 1. Amino acid replacements and their probable codon assignments at the A46 site (position 210) in the A protein of tryptophan synthetase (*1, 2*).