Transformation which occurred following infection with SA7 and SV20 met established criteria for the transformation process, *i.e.*, (a) cells grew in colonies and had a more epithelial-like appearance rather than the usual fibroblast-like appearance of newborn hamster kidney cells; (b) upon subculture, the cells showed an increased growth rate, and after forming a monolayer continued to divide and grew in masses or aggregates of cells; (c) upon inoculation into hamsters tumors appeared in the majority of the inoculated animals; (d) extracts of the cells contained a new T-antigen which was virus specific in the complement fixation reaction; (e) the hamsters in which tumors were induced by these cells developed complement-fixing antibodies to these antigens.

The cross-reactions in the complement fixation tests were not totally unexpected. The two simian adenoviruses (SV20 and SA7) produced antigens in the transformed cells which would react with some of the sera derived from hamsters whose tumors had been induced by cells transformed by the human adenovirus type 12. This was most evident in the case of the SV20 transformation, with 11 of the 12 individual sera reacting to some degree. These results suggest that with transformation, a spectrum of antigens is induced in the transformed cells, of which one or more are shared by the different oncogenic adenoviruses.

Studies are under way in our laboratory to determine the transforming titer of these simian adenoviruses, and whether different clones of these cell lines vary in their ability to produce complement-fixing T-antigen(s).

Summary. Two simian adenoviruses, SA7 and SV20, induced transformation of newborn-hamster kidney cells *in vitro*. The transformation was characterized by an increased growth potential of the cells, a change in morphology of the cells, the production of a new T-antigen within the transformed cells, the production of tumors in hamsters by inoculation of the cells, and the appearance of complement-fixing antibodies to T-antigen in animals which developed tumors. These new T-antigens are virus specific.

The excellent technical assistance of Mrs. Inta Ziedins is gratefully acknowledged.

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Pathogenic Properties of Encephalomyocarditis Virus Plaque Variants.* (32575)

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Two variants (r^+ and r) of the encephalomyocarditis (EMC) virus were isolated and characterized by Takemoto and Liebhaber(1). After 4 days of incubation under agar, plaques of the r^+ variant were 1 mm or less in diameter whereas those of the r variant were 8 mm or greater. The small size of the r^+ plaques was shown to be due to a sulfated acid polysaccharide in the agar overlay which bound the virus electrostatically and prevented spread to neighboring cells(2). As might be expected, agar extracts, heparin and synthetic sulfated acid polysaccharides such

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as dextran sulfate inhibited growth of the r^+ variants when they were incorporated in the liquid medium of cell cultures. The r variant was insensitive to these substances at equivalent concentration.

During studies to compare the properties of various EMC strains(3) it was found that the r^+ variant was non-pathogenic for adult mice when introduced by the subcutaneous (sc) and intraperitoneal (ip) routes but was highly virulent when inoculated intracranially (ic)(4). In contrast, the r variant and several other "wild" EMC strains were pathogenic when injected by all three routes. This report expands on these observations and records studies on the growth properties in tissues and mode of dissemination of the r^+ and r variants in mice.

Material and methods. Virus. The r^+ and r variants of EMC, supplied by K. K. Takemoto, were plaque-purified two times before stock pools were prepared. T. G. Akers provided the ME strain of EMC. Titrations were carried out in L-929 cell monolayers using a 1% Noble agar (Difco) overlay and 35 mm plastic dishes (Falcon). Plaque counts were made by neutral red staining 48 and 72 hours after inoculation(3). In some experiments titrations were done in tube cultures of L cells using cytopathic effect to determine end-points.

Animal studies. Male, 3- and 12-week Swiss mice were inoculated ic under ether anesthesia or sc in the lateral abdominal wall without anesthesia, using 0.03 ml and 0.1 ml volumes, respectively. Non-anesthetized suckling mice of mixed sex were injected with 0.03 ml aliquots by both routes. Brain was excised and mortar ground in diluent to make an approximate 10% suspension. The homogenate then was clarified by centrifugation. Skin and tissue from the abdominal wall surrounding the injection site were excised widely *en bloc* and mortar ground with sand in 3 or 5 ml of diluent. Coarse debris was removed by low speed centrifugation and the supernate recentrifuged at 10,000 $\times$ *g* for 15 minutes. Blood was obtained from the retro-orbital sinus and diluted 1:10. The clot was separated after 2 to 4 hours. A diluent made up of 5% heated (56°C for 30 minutes) chicken serum and Hanks' balanced salt solution was used routinely.

Tests for serum antibody and interferon. Neutralization tests were carried out in L cell monolayers using 2-fold dilutions of heated serum, a 50% plaque reduction end-point, and 50 to 75 PFU of the r^+ variant. Hemagglutination-inhibition tests were carried out as previously described(5). Tissues from 3 or 4 mice were homogenized in balanced salt solution (3 ml per animal) and the suspensions prepared for interferon assays as reported elsewhere(6).

Results. Revertant formation in L cells. Takemoto and Liebhaber found that small numbers of r revertant plaque forming units (PFU) appeared when the r^+ variant was grown and passaged in L cells under liquid medium(1). Our observations on revertant formation in r^+ infected cultures are presented initially since it will be shown below that reproducible results in animal studies are obtained only when a uniform population of r^+ PFU is employed.

Two distinctly different revertant PFU were recovered from cultures inoculated with the r^+ variant. One formed plaques identical to those of r; plaques of the second were intermediate in size (diameter 2-5 mm) between r^+ and r. This latter revertant (r^i) was purified readily. Like the r variant, it was insensitive to concentrations of dextran sulfate which inhibit growth of r^+(1). The r^i and r revertants invariably were present in virus pools prepared from cultures inoculated with both concentrated and dilute preparations of r^+. The ratio of revertant to r^+ particles (0.5—2%) varied somewhat in different experiments, but was roughly the same during single cycle (Fig. 1) or multi-cyclic (Fig 2) growth and after serial passage of undiluted preparations of r^+ in L cells.

Pathogenicity. The results of titrations of r^+, r^i and r pools in tube cultures of L cells and mice are summarized in Table I. Each of the variants was pathogenic for newborns when introduced by the i.c., s.c. or i.p. routes and infectivity titers were equivalent to those obtained in cell cultures. The titers in 3-week mice after i.c. injection also were identical to the *in vitro* results. However, r^+ titers,

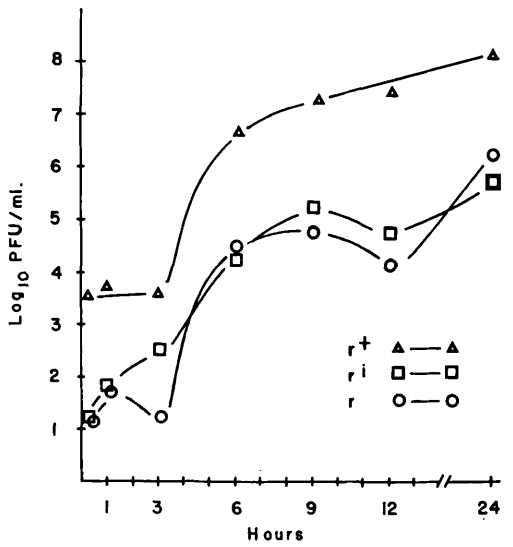

FIG. 1. Growth of r^+ variant and formation of revertants in L cells (input multiplicity = 4 PFU per cell). At indicated times 4 replicate cultures were frozen and thawed 2 times. Medium from each was titrated in agar overlaid monolayers.

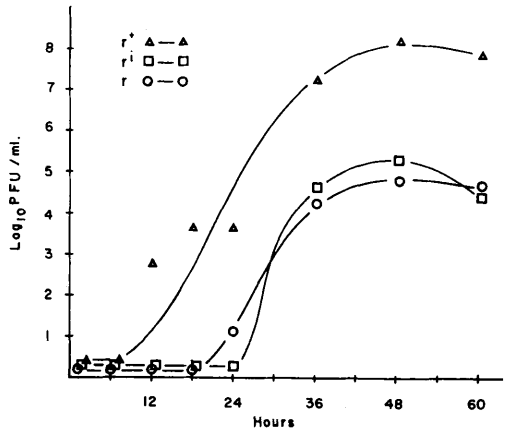

FIG. 2. Growth of r^+ variant and formation of revertants in L cells (input multiplicity = 10^{-7} PFU per cell). At indicated times 4 replicate cultures were frozen and thawed 2 times. Medium from each was titered in agar overlaid monolayers.

but not those of r^i and r, were substantially lower in s.c. or i.p. inoculated young adults. Additional studies were carried out in 12-week mice. Although mortality was variable in animals of this age, the results in Table I are representative of several experiments. Infectivity titers equal to those in cell cultures were obtained in the older mice only when r variant was introduced by the i.c. route.

TABLE I. Infectivity Titers of r^+, r^i and r Variants in L Cell Cultures and Mice of Different Ages.

Test system	Route	Variant inoculated r^+	r^i	r
L cells		8.0	8.0	6.0
2-day mice	ic	8.6	≦8.0	≦6.0
	sc	8.0	≦8.0	≦6.0
21-day mice	ic	7.8	8.2	6.5
	sc	5.0	8.0	6.0
12-wk mice	ic	5.7	ND	6.3
	sc	4.5	ND	3.8

Decimal dilutions of virus introduced into tube cultures of L cells or groups of 6 to 10 mice. Titers were calculated by the method of Reed and Muench using cytopathic effect or mortality to determine endpoints and are expressed as reciprocal $\log_{10}$ per ml. ND = Not done.

Detailed studies were undertaken in 3-week mice to account for the attenuated pathogenicity of the r^+ variant after peripheral inoculation. Two possibilities seemed likely from the available data: *a*) a minimum number of r^+ PFU (approximately 10^2-10^3) are required to initiate a disseminated infection after s.c. injection or *b*) the pathogenic viruses in more concentrated inocula are the contaminating r^i and r revertants which are present at low, but not high, dilutions of the virus pool.

Repeated parallel titrations of r^+ in animals and plate cultures showed that lethal infections developed only when one or more r^i or r PFU were present in the inoculum. Animals receiving a virus population comprised solely of r^+ (10 to 75 PFU) failed to develop viremia and illness whereas those given greater numbers of r^+, and accordingly varying numbers of revertants, experienced a prompt viremia and died with the neurologic syndrome characteristic of EMC. Since only r^i and r were recovered from the blood and brains of these latter animals, the revertants appeared to be the virus particles in the inoculum which disseminated.

Virus growth in tissues and viremia. To evaluate these findings, studies were carried out in 3-week-old mice to determine the fate of the r^+ and r variants after s.c. inoculation. The soft tissues of the lateral abdominal wall surrounding the injection site and the blood serum were assayed for virus

at 24-hour intervals. Replication of r^+ occurred in the tissues when dosages as small as 8 PFU were inoculated. Substantial amounts of virus were recovered from animals receiving 10 or more PFU (Fig. 3).

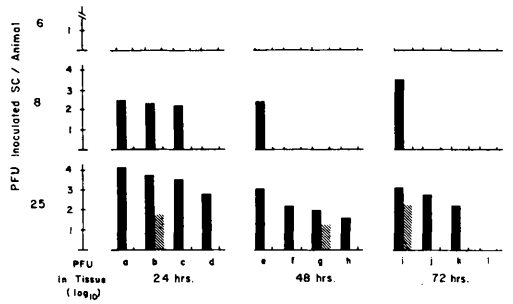

FIG. 3. Recovery of virus from tissues surrounding the injection site at intervals after sc inoculation of 3-week mice with r^+ variant. Solid bars = r^+; lined bars = r^1. Groups of animals were given a calculated 6, 8, or 25 PFU. Specimens from 4 mice (a - d) were obtained at each indicated time and titered individually in L cells under an agar overlay. Blood specimens obtained at time of sacrifice failed to yield virus.

The height of the virus titer and the duration over which virus was present in the tissues seemed to be related to the number of PFU in the inoculum. Although a few revertants occasionally were found in the tissues, virus failed to enter the blood at least in detectable amounts and could not be isolated from regional lymph nodes or major viscera. Similar results were obtained with 12-week animals.

The r variant also multiplied locally after s.c. introduction of small dosages of virus. However, the blood yielded r^1 and r PFU within 24 to 36 hours after inoculation (Fig. 4). Viremia in 3-week adults was invariably followed by fatal polioencephalitis. In contrast, older animals died only sporadically despite viremic periods of as long as 96 hours.

Virus growth in nervous tissue. The r^+ variant grew in the brains of 3-week-old mice after s.c. introduction of 5 PFU and was the predominant virus in the tissue at the time of death. The growth curve of r^+ in brain was similar to the curve determined in L cells using an inoculum of comparable size (Fig. 2) and the number of revertants formed were roughly equivalent. The r variant also multiplied promptly in nervous tissue after

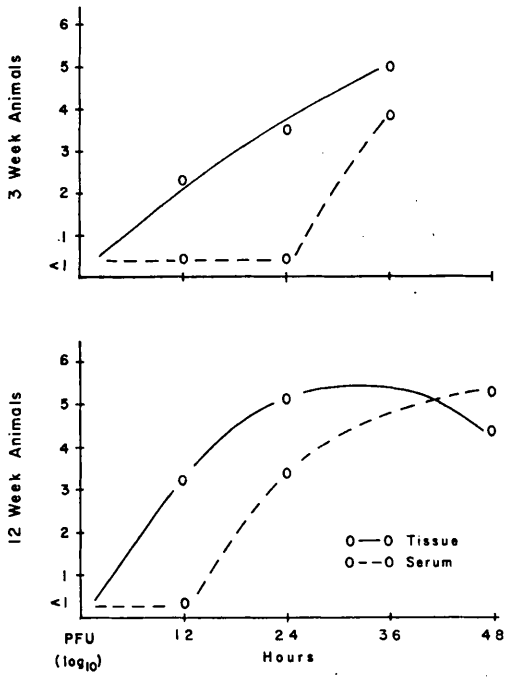

FIG. 4. Recovery of virus from tissues and blood at intervals after sc inoculation of 3-week (8 PFU per animal) and 12-week (12 PFU per animal) mice with r variant. Points indicate sum of r^1 and r PFU in tissue or serum.

i.c. injection. Although r appeared to replicate at a somewhat faster rate than r^+ in brain, differences in growth curves between the two variants were not striking.

Shown in Table II are results of studies in mice of 3 ages injected either i.c. or s.c. with approximately 10^5 PFU of r^+. As would be expected, the inoculum given these animals

TABLE II. Virus Recovered from Brain Tissue After ic or sc Inoculation of 10^6 PFU of r^+ Variant.

Animal age	Route of inoc.	Day tested*	Variant recovered r^+	r^1	r
2 day	ic	1	1×10^7	4×10^6	10^3
		2	1×10^6	1×10^7	1×10^6
21 day	ic	2	2×10^6	3×10^5	10^2
	sc	2	10^3†	2×10^6	10^3
12 wk	ic	6	10^3	2×10^6	10^3
	sc	6	10^4	7×10^7	10^4

* Animals sacrificed when ill or moribund.

† Detection of this type of PFU at concentrations lower than indicated was not possible because plaques of predominate virus population were confluent.

contained a substantial number of contaminating r^i and r revertants. Replication cycles of r^+ were limited after i.c. inoculation; the virus in the nervous tissue of fatally-infected animals exceeded the input by only 10- to 100-fold. Nonetheless, large numbers of revertant particles were found in the brain at death and they were the only PFU recovered from 12-week-old mice. After s.c. inoculation, a heterogenous population of PFU was present in the nervous tissue of moribund suckling mice but r^i and r predominated. Only r^i PFU were recovered from adults after s.c. inoculation. Thus, selective pressures seemed to favor the accumulation of r^i and r revertants in the brain regardless of the route of inoculation.

Immunologic response of 3-week-old mice injected s.c. with r^+ was investigated to determine whether or not local virus replication in tissues served as an antigenic stimulus. Serums from 12 animals bled 4 weeks after the injection of 50 PFU were examined for neutralization and hemagglutination-inhibition antibodies. Virus studies on appropriate controls during the first few days after inoculation showed that these mice had sustained a tissue infection. Neutralization titers of 1:5, 1:20 and 1:20 were obtained with 3 of the 12 specimens; the remaining sera were negative at a dilution of 1:5. Hemagglutination-inhibition antibodies were not demonstrated in the specimens at a dilution of 1:10.

Protection tests were carried out in 9-week-old mice which had been infected with 50 PFU of the r^+ variant 6 weeks previously. Animals were divided into groups and challenged either i.c. or s.c. with varying amounts of the pathogenic r variant (Table III). A low order of protection was demonstrated after s.c. challenge with 25 PFU of r. Mortality in animals injected i.c. with 25 PFU or s.c. with 250 PFU was similar to controls.

Interferon production. Sera and tissues from the injection site were assayed for interferon at intervals after the s.c. inoculation of r^+ and r variants. Interfering activity was demonstrated only in the tissues of r infected mice 72 hours after inoculation; however, the low concentration precluded detailed studies to determine whether or not interference was due to interferon.

Attempts to demonstrate interference *in vivo* were carried out by challenging r^+ infected mice with a lethal dose of r. Ten PFU of r were introduced s.c. into mice which had received 50 PFU of r^+ in the contralateral abdominal wall 24 or 48 hours earlier. These experiments failed to demonstrate protection attributable to the local r^+ tissue infection.

Interferon and antibody sensitivity. The r^+ and r variants were equally sensitive to interferon produced in the brains of Sindbis virus-infected, 8-day mice. Neutralization titers were comparable when the variants were tested in parallel against an antiserum prepared from rats which had been infected with the mengo strain of EMC.

"Wild" virus in cell culture and mice. Takemoto and Liebhaber considered the r^+ variant to be the "wild" plaque form of the EMC strain used in our studies(1). As reported by these workers, r^+ multiplies at a somewhat faster rate than r in L cells (Fig. 5). In addition, we have found that it produces 8- to 10-fold more virus per infected cell (Table IV).

A series of passages in L cells was initiated with each of the two variants by transmitting repeatedly, undilute fluids from cultures exhibiting cytopathic effect. As might be expected from the data in Fig. 5 and

TABLE III. Results of Mouse Protection Tests 6 Weeks After sc Inoculation of 50 PFU of r^+ Variant.

Dose and route of r challenge	No. survivors/No. tested	
	r^+ infected	Control
25 PFU ic	4/26	1/21
25 PFU sc	24/27	4/19
250 PFU sc	13/29	4/17

TABLE IV. Virus Yield by r^+ and r Infected L Cells.

Inoculum	Input multiplicity (PFU/cell)	Yield (PFU/cell)
r^+	30	3.8×10^8
r	27	4.5×10^2

Virus was adsorbed for 60 min at 20 C. Cultures then were washed 5× and harvested after incubation at 36 C for 12 hr.

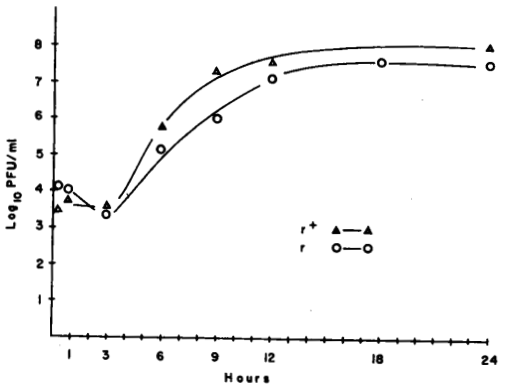

FIG. 5. Growth of variants in L cells (input multiplicity $r^+ = 2$, $r = 4$). At indicated times 4 replicate cultures were frozen and thawed 2 times. Medium from each was titered in agar overlaid monolayers of L cells.

Table IV, r^+ PFU predominated in the cultures after 20 passages of both the r^+ and r lines. Serial passages in 3-week-old mice also were initiated with the 2 tissue culture grown variants using the s.c. route of inoculation and virus from the brains of moribund animals. This work confirmed the results in Table II for it again showed that r^i and r PFU were selected during virus dissemination. Thus, the "wild" form of the virus strain reflected the system in which it was maintained.

Discussion. In evaluating the data presented here it is important to realize that revertants invariably were found in our stock pools of both the r^+ and r variants. Since r^i and r were highly pathogenic, results with the r^+ variant were affected dramatically by the inclusion of only a few revertant particles in an inoculum. Accordingly, doses of less than 100 PFU were used routinely in animal studies to assure a uniform population of r^+. The presence of r^+ revertants in r pools did not present a similar problem inasmuch as r^+ appeared to have no effect on r pathogenicity.

Three-week-old mice were employed in the majority of our experiments because differences in pathogenicity between the two variants readily were apparent in animals of this age. Both viruses multiplied in the brain after i.c. injection and regularly caused fatal neurologic disease. They also grew in soft tissues surrounding the site of injection. However, only r gave rise to viremia and a disseminated infection after peripheral inoculation. It would appear that r^+ failed to spread from the local site of replication since virus was never isolated from the blood, reticuloendothelial organs or nervous tissue.

The pattern of virus dissemination differed in newborn mice. Sucklings developed an r^+ viremia after s.c. inoculation and similar PFU were recovered from their brains before death. Nonetheless, r^i and r PFU predominated in the nervous tissue. Thus revertants appeared to be preferentially selected even though r^+ disseminated.

Twelve-week-old mice were relatively resistant when inoculated peripherally. Both variants grew in tissues at the injection site and viremia developed promptly in those animals receiving r. Nonetheless, central nervous system involvement occurred only sporadically. It would appear either that blood virus titers were too low to infect the brain or that a barrier between blood and brain existed in the older mice.

It is unclear from the data thus far accumulated whether or not individual cells of the intact animal favor the replication of r^i and r. If such is the case, growth of the variants in mouse cells occurs with contrasting efficiency *in vivo* and *in vitro*. It seems more likely that influences external to the cell affect virus spread in tissues and from one organ to another. Entry of r^+ into small blood vessels and lymphatics would appear blocked in adult mice. There is no evidence to indicate that r^+ particles are removed from the circulation with a higher degree of efficiency than r^i and r. Interferon or antibody produced during the early stages of the infectious process do not seem to significantly inhibit growth or dissemination of the viruses.

The r^+ variant is bound by naturally-occurring sulfated acid polysaccharides *in vitro*. These substances are intrinsic components of connective tissue(7,8) and are found in abundance within the peritoneal cavity(9,10). It is tempting to suggest that the sulfated acid polysaccharides of the connective tissue ground substance bind r^+ particles after s.c. or i.p. inoculation and physi-

cally or electrostatically restrict spread of the virus from cell to cell or to lymphatic and vascular channels. At present, experimental evidence to support such a hypothesis is lacking.

Small plaque variants of a number of picornaviruses have been described(11-15). Detailed studies of several of these viruses have shown that plaque size is related to the sensitivity of the virus to sulfated acid polysaccharide inhibitors in the agar overlay. Attenuated virulence of poliovirus type 1 vaccine strains (LCc 2 ab, Baylor Att-1) and certain foot-and-mouth disease vaccines also are associated with inhibitor sensitivity(12,16). It would be of interest to know whether or not these properties are biologically related or are independently occurring genetic variables. The EMC-animal system described here would appear to be a convenient model for further study of this relationship.

The pathogenic and plaque-forming properties of several different EMC strains have been investigated in this laboratory(3). A number of these viruses form plaques identical in size to those of r^+ but with the exception of the ME strain are virulent when introduced by peripheral routes. Although growth of each of these viruses is inhibited by agar extracts, the r^+ variant is more sensitive than any other strain thus far studied(3). These viruses also differ from r^+ in that they retain characteristic plaque-forming properties during passage in animals and fail to form obvious revertants(3). The ME strain(17) is comprised of a predominant population of non-pathogenic small PFU and a few pathogenic large PFU. Thus, this virus appears to be similar to the strain of EMC employed here.

Summary. The pathogenic properties of two plaque-variants (r^+ and r) of the EMC virus were investigated in mice of different ages. Both variants caused lethal infections in newborn animals after i.c., i.p. and s.c. inoculation. They also were virulent for 3-week mice when introduced i.c. However, the r^+ variant was non-pathogenic in adult animals injected by peripheral routes. The two variants grew for extended periods of time in soft tissues at the s.c. site of inoculation. Although viremia and central nervous system disease appeared in animals receiving r, a generalized infection failed to develop in those inoculated with r^+. No evidence was found to indicate that interferon or antibody altered the pathogenicity of this variant. Revertant PFU invariably developed when r^+ variant was grown in cell cultures; virulent r or r-like viruses comprised 0.5 to 2% of the PFU in r^+ pools. Accordingly, it was necessary to employ fewer than 100 PFU of r^+ to obtain a uniform population of the variant for pathogenicity studies. When larger dosages were introduced s.c. into animals, revertants disseminated and caused a lethal generalized infection. Growth of r and r-like revertants was favored in animal tissues and they were selected during serial passage *in vivo*. Since r^+ variant multiplied in L cells at a faster rate and produced more PFU per cell than r, passage of virus in cell cultures yielded a predominant population of r^+. Thus, the "wild" form of this strain of EMC virus differed according to the system in which it was maintained.

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Rise in Serum Immunoreactive Glucagon After Intrajejunal Glucose in Pancreatectomized Dogs.* (32576)

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Bioassayable glucagon has been reported to be present in the gastrointestinal tract and pancreas of man and other animals(1,2) but a different immunological behavior of intestinal and pancreatic glucagon has been shown (3,4). A rise of serum immunoreactive glucagon (IRG) has been demonstrated after alimentary glucose(5,6). Ohneda *et al*(6) reported that the rise in IRG after alimentary glucose was of enteric origin, but Samols (3) considered it was derived from the pancreas. Because it is crucial to know the relative contribution of the intestinal and pancreatic IRG to serum IRG level under various conditions, we have studied serum IRG in pancreatomized dogs under basal conditions and following alimentary glucose.

Methods and materials. Immunoreactive insulin (IRI) was assayed by the double antibody technique of Morgan and Lazarow (7). IRG was assayed by a sensitive double antibody technique based on the same principles as the insulin assay. Trasylol (Bayer Ltd.) was added to the incubation media in the glucagon assay (1000 Kallikrein inhibitor units per 300 μl serum) to prevent the degradation of labelled and unlabelled glucagon by serum(8). Glucagon antibody was raised in guinea pigs and used in a final concentration of 1:1875 in the assay, and crystalline beef-pork glucagon (Eli Lilly and Co.) was used as the standard reference in the assay. Details of the glucagon assay method are in preparation for publication. IRG was extracted from tissues by the method of Kenny (9). Plasma glucose was measured in the Technicon Autoanalyzer. Mongrel dogs, male Wistar rats and a squirrel monkey were used in the studies.

TABLE I. Immunoreactive Glucagon in Tissues.

	mμg/g wet organ			
	Human	Dog	Monkey	Rat
Pancreas	3700 (1)	2200 (3)	2500 (1)	275 (6)
Stomach	—	0 (2)	—	
Duodenum	—	0 (2)	—	}
Jejunum	290 (1)	43 (3)	—	} 44 (3)
Ileum	—	—	—	}
Colon	—	99 (2)	—	}
Rectum	120 (1)	—	—	} 58 (3)
Adrenal	—	0 (1)	—	—
Kidney	—	0 (2)	—	0 (1)
Liver	—	0 (1)	—	0 (1)
Spleen	—	—	—	0 (1)
Heart	—	—	—	0 (1)
Diaphragm	—	—	—	0 (1)
Thymus	—	—	—	0 (1)

No. of organs assayed is shown in parentheses.

Results. Table I shows that high levels of IRG were detected in the pancreas of 4 species of animals, and that the intestine contained smaller amounts, detectable from the jejunum to the rectum.

Fig. 1 demonstrates the inhibition slopes(10) of extracts of human intestine and pancreas. The human pancreas and rectal inhibition slopes and those of the monkey and dog pancreas (not shown), were all parallel to the beef-pork standard, suggesting their immunological identity. However, the human jejunal and dog jejunal slopes were signifi-

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