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Animal "Orphan" Enteroviruses

SEYMOUR S. KALTER, Ph.D.[1]

Since the discovery, some ten years ago, of the pathogenic effect of polioviruses on non-nervous-tissue cells, tissue-culture methods have come to be widely used in virological research. Through these improved techniques for studying viruses, a large number of new cytopathogenic agents have been isolated from the intestinal tract of man. Many of these agents have been obtained from persons suffering from polio-like disease, but others have been isolated from apparently normal persons. The term "orphans" is used to designate those viruses which cannot definitely be associated with any recognized disease syndrome.

The existence of these enteric pathogenic human orphan (ECHO) viruses, and their association with clinical disease in certain cases, stimulated interest in their animal counterparts, which might constitute a serious threat to both human and animal health. In this paper, the author reviews the information at present available on the occurrence of the so-called "orphan" enteroviruses in monkeys, cattle, swine, and other animals in various parts of the world, and discusses the possible interrelationships of these animal viruses with each other and with the human enteroviruses.

INTRODUCTION

> "... at every stage in scientific development, it is necessary to provide the best available generalizations as a guide to effective work, both in the application of knowledge to human needs and in the planning of future research."
>
> *F. M. Burnet*

Approximately ten years have passed since Enders, Weller & Robbins (1949) demonstrated the susceptibility of non-nervous-tissue cells to polioviruses and the production of a cytopathogenic effect (CPE) by such viruses. Prior to this development, virus studies were handicapped by the lack of an experimental host less complex than the monkey or the mouse. Thus, the introduction of tissue-culture methods to virological research led to rapid and epoch-making discoveries, unparalleled in recent microbiological investigations. Subsequent studies with tissue-culture systems have resulted in the discovery of vast numbers of cytopathogenic agents in many tissues and specimens submitted for virus examinations.

This exploration into new fields was so rewarding that an "excess" of viruses became apparent. Nowhere was this more evident than in isolations of agents from faecal material examined for evidence of poliovirus. The increased ease of isolating viruses from the intestinal tract of man, as well as the numbers of viruses isolated, made it clear to investigators that certain of these viruses did not belong to recognized poliovirus or Coxsackie-virus types. Whereas many of these agents were obtained from cases of polio-like disease (aseptic meningitis), similar agents were also isolated from normal persons. For want of a better name, and because of our lack of knowledge, the term "orphans" came to be commonly used for these agents when they could not be associated with disease. The number of hitherto undescribed isolated agents increased so steadily that a conference entitled "Viruses in Search of Disease" (New York Academy of Sciences, 1957) was arranged in order to ascertain which of these agents were responsible for recognized clinical disease.

The association of these organisms with the alimentary tract of man and the production of a CPE, when placed in tissue culture, prompted the term "ECHO (enteric cytopathogenic human orphan) virus" (Committee on the ECHO Viruses, National Foundation for Infantile Paralysis, 1955). Recognition that ECHO viruses had certain properties in common with polioviruses and Coxsackie viruses suggested the currently employed name of *entero-*

[1] Chief, Enterovirus Laboratory, Communicable Disease Center, Public Health Service, US Department of Health, Education, and Welfare, Atlanta, Ga., USA

viruses. The Committee on the Enteroviruses (1957) —formerly the Committee on the ECHO Viruses— recommended that, to be included among the enteroviruses, a virus should: (*a*) inhabit the alimentary tract of man; (*b*) be cytopathogenic for monkey and human tissue-culture; (*c*) have no relationship to other groups of viruses that may be recovered from the throat or intestine of man (for example, adenoviruses, influenza and herpes simplex viruses, etc.); (*d*) cause infection in man as indicated by the presence of neutralizing antibodies in human globulins as well as in an individual's serum from which the agent was isolated; (*e*) cause an intermingling of pathogenic effects in tissue-culture, suckling mice and monkeys (for example, ECHO 9 and 10 induce lesions in suckling mice similar to those produced by Coxsackie viruses; Coxsackie A7 and A14 and some of the ECHO viruses produce neuronal lesions in monkeys; etc.); and (*f*) have a characteristic size, resistance to ether, seasonal prevalence and epidemiological pattern. This has resulted in the recognition of 51 distinct viruses, with other candidates awaiting acceptance. Human enteroviruses sharing these characteristics consist of the following groups:

1. *Polioviruses*. Three types (1-3) which are antigenically distinct but which may cross-react if heated or formolized. All three types are capable of producing the same general clinical picture in patients, i.e., paralytic and non-paralytic disease.

2. *Coxsackie viruses*. Twenty-four accepted types divided into two groups. *Group A* (types 1-19) induce lesions in the skeletal muscle of suckling mice or suckling hamsters. These types do not proliferate, or only with difficulty, in the usual tissue-culture systems. Type A9 and possibly A7 are exceptions as they may proliferate in monkey kidney tissue-cultured cells. *Group B* (types 1-5) produce lesions in the brain and other organs of the suckling mouse. All five types may be isolated in monkey kidney cells, and less readily in HeLa cells. Both Coxsackie groups are capable of producing varied clinical diseases. Whereas herpangina is commonly due to group A and pleurodynia to group B, both may cause an aseptic meningitis and, more rarely, paralytic disease (Hammon et al., 1958; Kalter, Page & Suggs, 1958).

3. *Enteric cytopathogenic human orphan (ECHO) viruses*. In 1957, the Committee on the Enteroviruses reported 19 antigenic types. Since that report, at least five more types (types 20 through 24) have been included and a number of others are under study for acceptance (Melnick, 1958). The disease-producing capabilities of these ECHO viruses are not clearly defined. It is generally conceded that most strains have the capacity to cause an aseptic meningitis syndrome. It is quite probable that certain strains are or have been recognized as more frequent offenders in this clinical syndrome than others. The paralytic potential of these viruses has also been suggested (Hammon et al., 1958; Kalter, Page & Suggs, 1958). Exanthemas have been noted following infection with at least three of the accepted ECHO viruses. Whether or not this rash-producing characteristic indicates a common relationship requires further study. More recently, the association of ECHO viruses with infant diarrhoea has been indicated (Ramos-Alvarez, 1957; Ramos-Alvarez & Sabin, 1956, 1958; Eichenwald et al., 1958). The capacity of ECHO 9 virus to produce a myositis and paralysis of suckling mice is similar to that observed following infection with the Coxsackie group-A viruses. This would suggest the possible reclassification of this virus with the Coxsackie viruses rather than with ECHO viruses. Similarly, the position of ECHO 10, in the present classification, is not clear. This agent is larger than other members of the group, has a distinctive CPE and apparently produces a respiratory-type disease in chimpanzees and children (Sabin, 1957). It was also demonstrated that certain of these ECHO 10 viruses produced lesions in suckling mice characteristic of the Coxsackie group-B viruses, i.e., necrotic lesions in the liver and cardiac muscle. The ubiquitous nature of ECHO viruses may even extend to causation of respiratory disease (Cramblett et al., 1957; Kalter, unpublished data). It is obvious that numerous unknown factors are involved in the development of strain and type differences. For example, " prime " strains were reported for many of the ECHO viruses with broad antigenic characteristics. Antisera prepared against the " prime " strain may poorly neutralize strains of the " parent " type.

The existence of human enteroviruses [1] and their association with disease has resulted in a refocusing of attention on the presence of animal counterparts. The emergence of these animal enteroviruses as possible important challengers of human and animal health is now under serious consideration by a

[1] See recent reviews by Committee on the Enteroviruses, National Foundation for Infantile Paralysis, (1957); Melnick (1958); Sabin (1958); etc.

number of investigators. The purpose of this report is to review the available information regarding the presence of enteroviruses in various animal families, and to analyse these results with a view to formulating a basic concept regarding common interrelationships.

Appreciation of the existence of animal enteroviruses can hardly be said to be of recent origin. Animal diseases, their existence and possible transmission to man, have been of interest to numerous investigators for many years. One cannot disregard such examples as rabies, vaccinia-variola, arthropod-borne disease, etc., to cite only a few.

Martin (1958) has recently analysed the relationship of animal diseases to the health of man and states:

> " All groups interested in animal health and human health and welfare must join together in sharing attitudes, knowledge, information, skills, methods, and plans to eradicate the great animal plagues that affect man, and to share in the prevention of the problems of tomorrow that will result as man either adapts to or changes his environment."

The present use of tissue-culture methods may be considered responsible for the renewal of interest in animal enteroviruses. For the purposes of discussion, two viruses commonly isolated from the intestinal tract of mice may very well have been the first unrecognized animal enteroviruses. Would the isolation of lymphocytic choriomeningitis virus and Theiler's virus by our present tissue-culture methods have resulted in their classification as ECRO (enteric cytopathogenic rodent orphan) viruses (Traub, 1935; Theiler, 1937)?

Recent investigations have provided examples of isolations from the alimentary tract of many different animals. An attempt will be made here to discuss only those viruses currently considered " orphans " because of their inability to produce a particular disease. The " enteric cytopathogenic orphan " terminology is used throughout this review, improvising wherever necessary for the animal family involved. Certain new viruses, other than orphans, will be included whenever their recognition is considered necessary. The various isolates and responsible investigators are listed in Table 1.

A number of animal diseases are caused by viruses that may be found upon examination of faecal material. Of interest are the viruses found in animals which are not transmissible to man, but are capable of producing polio-like diseases, canine infectious hepatitis, and various gastro-intestinal infections (Koprowski, 1958). Discussion of these organisms is not included in this review.

GENERAL PROCEDURES

By definition, the various viruses under consideration were isolated in tissue-culture systems. The majority of laboratories employed similar methods of cell cultivation. Monkey kidney cells were generally used, either as the primary host cell or in conjunction with a specific host cell system—for example, bovine kidney, swine kidney, etc. An attempt to study and evaluate isolates in other cell lines—of human or animal origin—was frequently made. As the general procedure for culturing cell lines was similar, although no standardization of method has been attempted, the preparation of monkey kidney cells will be briefly described. Evidently, a basic growth-medium can be used for the cultivation of tissue cells from different animals with equal success (Madin, Andriese & Darby, 1957).

Monkey kidney cells were usually trypsinized by the method of Younger (1954). Minced kidney cells were extracted with 0.25% trypsin (Difco 1 : 250) in Hanks' balanced salt solution (BSS) and grown in medium 199 plus serum (10%) or lactalbumin hydrolysate plus serum (0.5% lactalbumin hydrolysate, 2% calf serum and 97.5% Hanks' BSS) and antibiotics (penicillin and streptomycin) were added to ensure sterility. Although roller tubes or suspended cell preparations were used by some, most laboratories appeared to prefer the stationary tube culture without the plasma clot. Maintenance media contained little or no serum. Kidney cells from other animals were prepared in a similar fashion. Plaque studies were not employed by most laboratories. The use of this technique, originally described by Dulbecco (1952), was modified by Hsuing & Melnick (1955) for use in bottles. These investigators used bottle cultures of trypsinized monkeycells in a growth medium of 0.5% lactalbumin hydrolysate and 2% calf serum for plaque formation. Infected cells were overlaid with 1.5% melted agar in Earle's salt solution, containing 2% calf or monkey serum and 0.0017% neutral red. After solidification of the agar, the bottles were inverted and re-incubated at 37°C.

Serological identification depended upon neutralization of the isolates with known antisera. Antisera were prepared in laboratory animals, primarily monkeys and rabbits, by usual methods and were

TABLE 1
ANIMAL ENTEROVIRUSES

Host	Isolated from:	Tissue-culture system	Number of isolates	Number of prototypes	Geographical situation	Investigators	Clinical disease
Cattle (ECBO)	Stools	Bovine kidney, monkey kidney	8	1	Central USA	Kunin and Minuse	None
	Stools	Calf kidney	26	3	Eastern USA	Luginbuhl	None
	Stools	Bovine kidney	9	2+		Moll and Finlayson	Respiratory disease
	Rectal swabs	Monkey kidney	11	2(?)	Central USA	Moscovici and Maisel	None
	Stools	Calf kidney ±monkey kidney	70+	6+	Eastern USA	Klein	None
	Stools	Bovine and monkey kidney	6+	?	Eastern USA	Abinanti	?
	Stools	—	—	1 (poliovirus type 1)	—	Koprowski and Norton	—
	Stools	Monkey and calf kidney	100+	3	Northern Ireland	McFerran and Dane	None
Swine (ESCO)	Stools	Monkey kidney, swine kidney	1	1	Italy	Moscovici and co-workers	Diarrhoea
	Stools	Swine kidney	376	1+	Central USA	Beran, Werder and Wenner	None
	Stools	Swine kidney	5	1	New Zealand	Webster	None
Monkey (ECMO)	Monkey kidney	Monkey kidney	4	1	USA	Rustigian, Johnston and Reihart	None
	Monkey kidney	Monkey kidney	1	1	USA	Enders and Peebles	None
	Monkey kidney, stools	Monkey kidney	?	?	USA *	Hull, Minner and Mascoli	None
	Rectal swabs, stools	Monkey kidney	169	13	Eastern USA	Hoffert, Bates and Cheever	151/176 diarrhoea 18/19 no »
	Monkey kidney, stools	Monkey kidney	7	4	South Africa	Malherbe and Harwin	None
	Stools	Monkey kidney	25+	3	USA	Riordan	Diarrhoea and normal
	Monkey kidney, stools	Monkey kidney	51	3+	USA	Hsuing and Melnick	?
Poultry (ECAO)	Stools	Chicken kidney	42	?	Eastern USA	Luginbuhl and Burke	None
Cat (ECCO)	Tissue	Kitten kidney	1	1	New Zealand	Fastier	None

A plus sign after the figure for the number of isolates or prototypes indicates that an additional number of isolates or prototypes may exist.

* A number of simian viruses have been received by Hull from other countries. Certain of these have been found to be related to those isolated in his laboratory. There are, however, a number that need identification.

used in neutralization tests against approximately 100 TCD_{50} of virus. The sera of animals from whom the isolate was obtained were tested for production of homotypic antibody. The serum dilution that prevented the virus from producing a CPE in 50% of the tissue-culture tubes was taken as the titre.

Faeces were the main source of material for virus isolation. Studies with monkeys often involved differentiating the agent in stools from that present in the kidney tissue. A 10-20% homogenate was prepared by suspending the stools in a flask containing a diluent—for example, saline, Hanks' BSS, etc. All investigators used antibiotics (penicillin, streptomycin and mycostatin) at various dilutions in the diluent. The stool suspensions were then centrifuged for different periods of time. In most instances, low-speed followed by high-speed centrifugation (10 000-14 000 revolutions per minute for 1 hour) was employed. Tissue-culture tubes were inoculated with 0.1-0.2 ml of suspension per 1.0 ml of fluid.

The recognition of the presence of an agent in tissue culture is dependent upon a recognizable CPE. This varies in intensity from a slight histopathological effect (rounding of cells, nuclear pyknosis, cellular fragmentation) to complete destruction and failure to adhere to the side of the glass tube (or bottle). Specific virus CPE is not seen, but the experienced investigator is often able to obtain a relatively good grouping of organisms by certain characteristics.

MONKEY (ECMO) AGENTS

In an attempt to adapt dengue virus (Hawaii strain, mouse-adapted) to monkey kidney-tissue cells, Rustigian, Johnston & Reihart (1955) encountered an unidentified agent which on transfer produced a CPE in monkey kidney and HeLa cells. Subsequent studies revealed three additional agents in monkey renal tissues. These findings were reminiscent of the earlier report of Enders & Peebles (1954) in which they described an agent isolated from uninoculated monkey kidney-cell cultures. Species differences evidently exist among monkeys in regard to the presence of these " foamy " agents. Hsuing & Melnick (1958) reported that African monkeys may differ from Asian monkeys in their failure to contain viruses of this type in kidney-cell preparations. Foamy viruses, although not considered enteroviruses, have been found in tissues extensively employed for virus isolations and constitute a major source of annoyance and virus contamination.

Hull, Minner & Smith (1956), while producing and safety-testing poliomyelitis vaccine, encountered numerous CP agents. These were separated as eight immunologically distinct viruses and designated " simian viruses (SV) ". Prototype strains were: SV 1, 2, 4, 5, 6, 11, 12, and 15. Of these eight agents, only two—namely, SV 2 and SV 6—were isolated from faeces. These two simian viruses may be responsible for disease as they were isolated from animals with diarrhoea. However, upon intramuscular inoculation, neither was able to produce indications of overt infection in monkeys. Two others, SV 12 and SV 16, caused paralysis and death following intracerebral inoculation into monkeys. The eight simian viruses produced a characteristic CPE (see below), allowing rapid identification and grouping. In addition, they were observed to be filterable through 03 Selas filters, capable of growth in Maitland cultures, and variable in their ability to grow in tissue cultures of monkey testis, HeLa cells, and human kidney. SV 2, 5, and 6 did not proliferate in any of these tissue cultures, nor were they destroyed by heating at 65°C for 30 minutes, as the other strains were. All eight prototypes were reduced in titre to 1 TCD_{50} in 96 hours or less after contact with 1: 4000 formaldehyde. In monkey kidney-cell cultures, infectivity occurred at dilutions of 10^{-6} to $10^{-7.5}$ TCD_{50}. Subsequent studies by Hull & Minner (1957) revealed the presence of an additional 13 agents, previously not categorized. These, plus agents isolated by other investigators, have added another 20 to the original eight strains (Hull, Minner & Mascoli, 1958). Two previously tested strains, SV 22 and SV 24, were dropped from the new classification. SV 22 proved to be identical with SV 23, whereas SV 24 was shown to be an amoeba. All current strains may be separated into one of the four CPE groups (see below) described by Hull, Minner & Smith (1956). These include an additional 24 agents submitted to Hull by other investigators. In addition to these " classified " viruses, there are others under study.

The characteristics of these new simian viruses have been studied by Hull, Minner & Mascoli (1958) as were the original eight strains. The infectivity of the new strains to monkey kidney cells was similar to that of the original strains except that SV 36 was low-titred ($10^{-3.5}$) and SV 26 and SV 27 were high-titred ($10^{-7.5}$ and $10^{-8.0}$, respectively). Inactivation by 1: 4000 formaldehyde readily occurred within 75 hours. Variable virulence for monkeys and mice was noted among these strains. Growth

in tissue-cell lines other than monkey kidney cells was limited: on HeLa cells by SV 33, 34, 36, and 59; human amnion by SV 25, 28, 36, and 59; and monkey heart by SV 27, 28, 34, 36, and 59.

Cross-neutralization among strains became more frequent as additional viruses were found. Very few cross-reactions, if any, occurred between CPE groups. SV 4 was often neutralized by normal rabbit serum. The CPE of these simian viruses differed when " grown in good quality trypsinized monkey kidney cell cultures ". Accordingly, Hull, Minner & Smith (1956) separated the viruses into four groups:

Group 1. Rounded cells, grape-like clusters and attached to the glass wall. The viruses in this group are similar to the adenoviruses in their CPE (SV 1, 11, 15, 17, 20, 23, 25, 27, 30, 31, 32, 33, 34, 36, and 37).

Group 2. Pleomorphic cells of greater density than normal cells with discrete cell membranes (SV 2, 16, 18, and 19).

Group 3. Spindle-shaped or normal cell morphology with granulation and slight vacuolization of cell cytoplasm often obliterating nucleus. These cells were often observed floating free in the medium except for one long process adhering to the glass (SV 4, 12, 28, and 59).

Group 4. A miscellaneous group. The CPE produced by SV 5 results in giant cells with many nuclei, resembling mumps and measles viruses. The CPE of SV 6 is similar to that seen in poliovirus-infected cells. SV 26 and SV 29 cause rounding of cells but without clumping or complete destruction. SV 35 produces an early polio-like CPE (resembling SV 6) followed by a group-2 effect (SV 5, 6, 26, 29, and 35).

This CP grouping not only serves to type various simian agents but also assists in: (*a*) safety-testing poliovirus vaccine by indicating the presence or absence of polioviruses, and (*b*) demonstrating the presence of several viruses in a mixture.

Other investigators have reported on the isolation of monkey orphan viruses. Cheever (1957), in a study of diarrhoeal diseases in primates, isolated a number of virus agents from monkey faecal material. Stools from animals with diarrhoea yielded virus agents in 86% of the stools examined. Normal monkeys also harboured large numbers of agents in their alimentary tracts. Many of these agents were related to recognized simian viruses while others apparently were new. Five agents were antigenically related to the adenoviruses by complement-fixation (CF) tests; one (M25) may be a new adenovirus. A more extensive report on these findings was made by Hoffert, Bates & Cheever (1958). Rectal swabs were examined from 176 monkeys with diarrhoea and 19 presumably normal animals. Virus agents were obtained from 169 (87%) of these specimens. Both groups of animals manifested approximately the same frequency of isolations. Two CPE groups similar to those described by Hull and co-workers were found. A similarity to adenoviruses, ECHO and Coxsackie viruses was noted. Antigenic analysis indicated the presence of 13 serotypes, designated P 1-13.

These viruses varied in infectivity for monkey cells from $10^{-4.5}$ to $10^{-7.0}$ per 0.25 ml. Adenovirus group CF antigen was found in CPE group-1 agents (P 4-10 inclusive). CPE group-1 viruses, when inoculated into HeLa cells, demonstrated their similarity to adenoviruses by producing the same characteristic CPE. Group-2 viruses did not produce a recognizable CPE in HeLa or in human amnion cells. All the group-1 viruses, except P 5, exhibited a CPE in human amnion cells on first passage. Group-1 CP activity resulted in slow changes, taking about ten days to affect all cells. Rounding, swelling, elongation, and development of refractile cells was typical. The effect of group 2 on monkey cells was more rapid, a rounding and granulation of all cells occurring within four to five days. Of the 13 strains, only P 2 and P 13 were pathogenic for suckling mice. These animals developed a paralysis followed by death. A generalized myositis was found upon histological examination and to a lesser extent involvement of the myocardium. These 13 viruses, when tested by Hull, Minner & Mascoli (1958), were reclassified into the SV number designated for this group (Table 2). Neutralizing-antibody studies on sera from animals with diarrhoea and without diarrhoea showed a consistently higher percentage of antibodies to all prototypes in healthy animals than in animals with diarrhoea.

Melnick (1957) refers to studies performed in his laboratory by J. T. Riordan. In this investigation, 25 ECMO isolates were made from stools in one series of 21 monkeys. It was also observed that some viruses were excreted for weeks after the first isolation was made. Riordan (personal communication, 1958) reported on studies completed with monkeys (rhesus and cynomolgus) during the years 1956-57. CP agents were readily isolated by means of rectal swabs from both normal animals and those ill with diarrhoea. With very few exceptions, the

TABLE 2
PRESUMPTIVE GROUPING OF SIMIAN VIRUSES ACCORDING TO PLAQUE MORPHOLOGY AND *PATAS* CELL SUSCEPTIBILITY *

Group A : small, delayed plaques on rhesus cultures and on *patas* cultures		
7852	SV 1	P 4
7853	SV 4	P 5
7854	SV 11	P 6
7855 A	SV 12	P 7
7999 A	SV 15	P 8
	SV 17	P 9
	SV 22	P 10
	SV 23	
	SV 25	
	SV 27	
	SV 28	
Group B : large, circular plaques with islets on rhesus cultures and on *patas* cultures		
7848	SV 2	P 1
7849	SV 16	P 3
7850	SV 18	P 11
7998		
8000		
8226 **		
8228		
8229 **		
8422		
8423		
8427		
8429		
Group C : large, circular plaques with islets on rhesus cultures, but no plaques on *patas* cultures		
7855 B	SV 6	P 2
7999 B		
8006		
8227		
8425		
8426		
8431		
8432		

* The strains listed in the column on the left were isolated in our laboratory (Hsuing & Melnick); the SV series in the centre column were made available by R. P. Hull, Lilly Research Laboratories, Indianapolis, Ind.; and the P series in the column on the right were supplied by F. S. Cheever, University of Pittsburgh, Pittsburgh, Pa.

** Mixed strains.

Reproduced, by permission, from Hsuing & Melnick (1958)

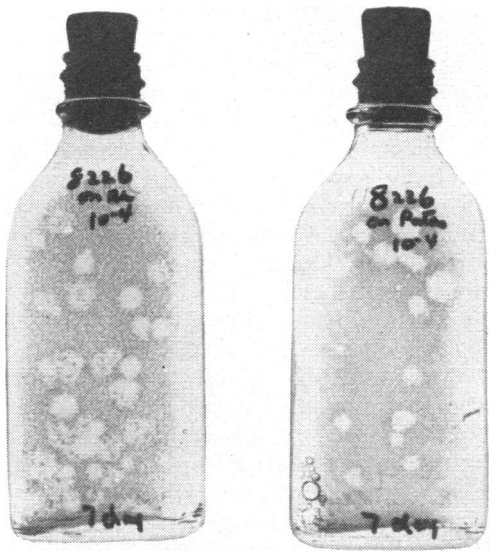
23 isolates could be divided into three groups on the basis of their CPE. A fourth group comprised mixtures of agents. Many of these agents were similar to those isolated by Hull and co-workers.

Similar findings have been reported by N. J. Schneider (personal communication, 1959) in a study on moribund animals. From 373 tissues inoculated into monkey kidney cell tissue-cultures, 118 CP agents were isolated. A number of these agents have been identified as related to previously described simian viruses. A small number (11) were pathogenic for suckling mice, but the remainder failed to cause discernible disease in these animals. The highest yield of organisms was derived from the kidney (41 isolations from 87 kidney examinations, i.e., 47.1%). The remaining isolations were made from faeces (44.4%), spleen (43.3%), heart (35.7%), lung (31.0%), plasma (14.8%), and pancreas (7.1%).

Hsuing & Melnick (1958) have attempted to group the simian viruses according to their plaque morphology and *patas* monkey kidney cell susceptibility (Fig. 1). This classification resulted in the three groups illustrated in Table 2.

Two suggestions have been made for classifying these monkey orphan viruses; one is based on

FIG. 1
ECMO VIRUSES (No. 8226) ON RHESUS (LEFT) AND *PATAS* (RIGHT) CULTURES SEVEN DAYS AFTER SEEDING



Two kinds of plaques were noted in the rhesus bottle cultures, some with and some without islets. Only plaques without islets were observed on the *patas* cultures.

Reproduced, by permission, from Hsuing & Melnick (1958)

rhesus monkey kidney type CPE and the other on plaques and *patas* monkey kidney cell culture. It is of interest to note that the general grouping of these various agents was similar, giving support to the separation of these viruses into distinct antigenic groups. This is substantiated further by the findings of Hull, Minner & Mascoli (1958) that cross-neutralizations were observed principally within the CPE group. This similarity of viruses would indicate that one system be employed for taxonomic purposes.

Hull suggested that all simian viruses in CPE group 2, as well as SV 6 of CPE group 4, be classified as monkey enteroviruses. These viruses were mostly isolated from stools and only rarely from other material. CPE group-1 viruses, which are related to human adenoviruses, may on occasion be isolated from stools, but Hull (personal communication, 1958) does not consider the alimentary tract as primary tissue for this group. CPE groups 3 and 4, with the exception of SV 6 (CPE group 4), have not been isolated from faecal material.

More recently, Hull, Minner & Mascoli (1958) have studied additional simian viruses submitted to their laboratory for classification. Their results indicate that new agents will apparently continue to be found among presumably normal monkeys. Most of the agents submitted were antigenically related to existing simian viruses and fell into one of the four CPE groups previously described. A few viruses showed no apparent relationship or were only remotely related to agents already studied.

Monkey agents have also been reported from other geographical areas where these animals were used. Malherbe & Harwin (1957) isolated seven viruses from vervet monkeys (*Cercopithecus aethips*), which are most commonly used for poliovirus work in South Africa. Of the seven strains, six were isolated from uninoculated cultures of kidney tissue and one from a stool sample. It was also shown that the SA 1 strain was similar to that described by Rustigian, Johnston & Reihart (1955); SA 3 was neutralized by ECHO 10 antiserum and the SA 7 strain found in the stool sample produced inclusion bodies.

The behaviour of these simian viruses in suckling mice may have an important bearing on the future classification of enteroviruses. Two strains described by Hoffert, Bates & Cheever (1958)—P 2 (Hull SV 19) and P 13 (not as yet classified by Hull, but in CPE group 4)—produce lesions in suckling mice similar to those seen following Coxsackie virus inoculation. Coxsackie antisera group A (7, 9, 11, 13, 14, 15, and 18) and group B (1-5) failed to show any relationship to these two isolates. The pathogenicity of P 2 (SV 19) for suckling mice was confirmed by Hull's group, as was the finding that P 5 (SV 30) was also infective for suckling mice. All of these were infectious only after intracerebral inoculation except SV 19, which caused infection after intramuscular inoculation. It might be expected that this vast number of agents would bear some relationship to other known viruses. This, however, is apparently not the situation, except in a few cases—a fact which suggests the possibility that the virus flora of each animal species may be characteristic of that particular species. Human agents may occasionally be present, as evidenced by the isolation of Asian influenza virus (Hull, Minner & Mascoli).

The findings with B virus have not been included in this review. This agent is frequently present in monkeys, as evidenced by a number of isolations and the frequency of antibody (approximately 60% rhesus and cynomolgus monkeys have B-virus antibody) (Hull, Minner & Mascoli).

BOVINE (ECBO) AGENTS

The isolation of numerous agents from monkey tissues and excreta is apparently duplicated in cattle. Only limited studies were made of tissue viruses, the major efforts being concentrated on stool isolations. An agent related to the psittacosis/lymphogranuloma-venereum group of viruses was isolated from the faeces and intestine of normal calves by York & Baker (1951). This organism was appropriately named *Miyagawenella bovis*. Other agents have since been isolated, but most were shown to be related to previously described viruses or associated with a specific disease syndrome.

The term ECBO viruses was suggested by Kunin & Minuse (1957, 1958) in reporting on eight strains of enteric viruses obtained from "healthy" dairy cattle. A similar observation was made by Klein & Earley (1957). These and other findings have since been reported by other investigators (Moll & Finlayson, 1957; Luginbuhl, 1958). The isolation of a type-1 poliovirus from calf faeces by Koprowski may be of importance, although he did not rule out the possibility of laboratory contamination.

The eight cytopathogenic agents isolated by Kunin & Minuse were all antigenically similar and were obtained from cattle on three dairy farms where there was no apparent illness among the animals.

All but one strain (NH F 9) exhibited a similar CPE in tissue cultures of bovine kidney and this finding led to the designation of one strain as the prototype (LC R 4). These viruses were readily adapted to monkey kidney cells, but not to HeLa cells. The cellular changes in tissue culture were similar to those seen after infection of monkey kidney cells with poliovirus. TCD_{50} titres per 0.25 ml inoculation were of the order of $10^{-6.0}$ in cattle kidney culture and about 1 log lower in monkey kidney cells. LC R 4 was passed through Seitz (with positive pressure) and Mandler No. 13 and No. 15 filters. The particle was small, having a sedimentation constant of about 150-200 Svedburg units. Shaking with an equal volume of ether did not reduce the titre after 26 hours, nor did storage at 4°C for 4 days. Storage in the presence of carbon dioxide in screw-capped tubes resulted in a loss of titre after a few weeks.

Evidence of disease could not be induced in monkeys, rabbits, or young adult mice (three weeks old), but the intraperitoneal inoculation of 24-hour-old mice resulted in paralysis and death, producing a pathological condition similar to that produced by the Coxsackie group-A viruses. Direct inoculation of a stool suspension into embryonated eggs (amniotic cavity) also resulted in virus isolation. Virus growth occurred throughout the chick embryo, causing a thickening of the chorio-allantoic membrane and production of pocks following membrane inoculation. Neutralization tests performed in both monkey kidney tissue-culture cells and in fertile eggs, using antisera prepared in rabbits, indicated an antigenic similarity (including strain NH F 9). The sera from several herds showed the presence of antibodies to these viruses, while poliomyelitis antisera failed to neutralize. Bovine virus antiserum prepared in rabbits was unable to neutralize polioviruses. In addition, no antigenic relationship could be demonstrated to the following viruses: Coxsackie group A, types 1-19, and group B, types 1-5; ECHO 1-14; vesicular stomatitis; calf pneumonitis; Sabin calf EC 25; and bovine rhinotracheitis. Sera from man and pigs failed to neutralize the agent. Strain NH F 9 is apparently a slow-growing prototype of the other bovine viruses.

W. N. Mack (personal communication, 1958) studied the agents isolated by Kunin & Minuse and confirmed the susceptibility of embryonated eggs, suckling mice and hamsters to the prototype strain (LC R 4) as well as the inability of that strain to produce apparent disease in adult mice, white rats, cotton-rats, guinea-pigs, cats, dogs, chickens, and one-day-old chicks. Pathogenicity studies demonstrated that this agent could be isolated from the stools of a 3-month-old calf for 13 days after intravenous inoculation, and that during this period the calf showed no overt symptoms but developed specific antibodies.

J. B. McFerran (personal communication, 1958) reported that over 100 CP agents were isolated from cattle faeces in Northern Ireland. These isolates fell into three immunological types, one of which was neutralized by antiserum prepared against Kunin's LC R 4 virus. These agents grew equally well in monkey kidney and calf kidney tissue-cultures.

Klein & Earley (1957) described the isolation of 35 viruses in calf kidney-cell cultures from four dairy herds. Most of these viruses, i.e., 33, were isolated from young animals (six months to two years old). The others were obtained from an adult animal and as a latent calf kidney tissue-culture virus. These agents could be isolated only in calf kidney cells and three distinct CPE groupings were noted. Poliovirus antisera failed to neutralize any of the isolates. As indicated previously, a large percentage of the animals possessed poliovirus-neutralizing substances in their sera. Subsequent studies by Klein (personal communication, 1958) have resulted in the isolation of over 70 strains from apparently healthy animals. These strains, which grow well in calf kidney cells but " rather poorly " in monkey kidney cells, may be separated into at least six distinct groups. Two strains were found to be neutralized by human gamma-globulin and a large number of individual human sera. Although the animals from which the strains were isolated appeared normal, increases in antibody to the isolate have been detected.

More recently, Klein, Earley, Frankel & Zellat (personal communication, 1959) reported on the isolation from faeces of a bovine virus neutralized by 35% of the human sera against which it was tested. Four different lots of human gamma-globulin neutralized this agent at a final dilution of 1: 320. Antisera prepared against adenoviruses 1-7, Coxsackie viruses B 1-5, ECHO viruses 1-19, haemadsorption viruses 1 and 2, influenza viruses (A—including A/Asian—, B, and swine), herpes simplex virus, B virus, mumps virus, and all three polioviruses failed to neutralize the agent. This bovine virus grew poorly in bovine kidney cells (10^{-3} TCD_{50} per 0.1 ml), though with the production of a

characteristic inclusion body, and did not grow in monkey kidney or HeLa cells, in adult or suckling mice or in chick embryos.

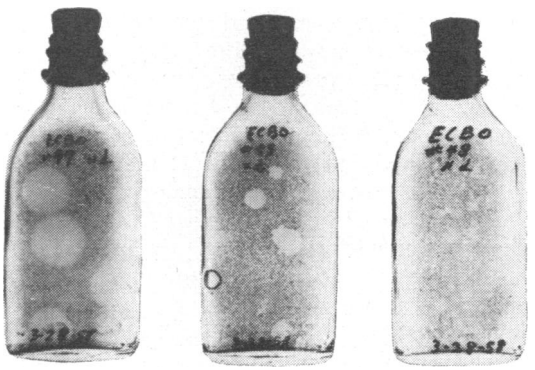
The isolation of ECBO viruses from 26 of 111 cattle was reported by Luginbuhl (1958). Seventeen isolations were made in calf kidney cells and 20 in monkey kidney cells. Where isolations were made in both systems, only one isolate failed to adapt itself to the other system. Three herds were studied: A, three years or older; B, ages 14 to 29 months; and C, ages 4 to 13 months. The majority of the isolations were from the youngest animals (herd C, 64.3%); herd B showed a frequency of infection of 15.1% and herd A failed to yield any agent. These data do not indicate whether this difference in frequency is due to age or merely to herd location, but the findings of Klein & Earley suggest that age may influence infection distribution.

The CPE of these viruses was similar to that of poliovirus. All isolates produced plaques, the majority (84%) forming mixed plaque types, i.e., large plaques and small plaques. On the basis of plaque formation, Luginbuhl designated three types: *type A*, approximately 10 mm in diameter after 7 days and containing some surviving cells which give a "hazy appearance"; *type B*, approximately 5 mm in diameter after 7 days and clearly outlined; and *type C*, very small plaques, i.e., about 1 mm in diameter after 7 days, with an opaque central core (Fig. 2).

Serological differences were also noted among the isolated strains. Attempts to associate these agents with those described by Kunin & Minuse failed to indicate any serological cross-reactivity, although a similarity in plaque type was demonstrated. On the other hand, one of the sera prepared against virus No. 58 did manifest a slight cross-reaction with the agent isolated by Moll & Finlayson (1957). Of interest was the observation that variations were encountered in the capacity of monkey pre-immunization sera to neutralize different isolates. The significance of this observation is not clear.

FIG. 2

PROTOTYPE ECBO PLAQUES A, B AND C (LEFT TO RIGHT) FORMED ON MONKEY KIDNEY CELLS BY ENTERIC CYTOPATHOGENIC AGENTS ISOLATED FROM CATTLE: ELEVEN DAYS AFTER INOCULATION



Photograph supplied through the courtesy of Dr R. E. Luginbuhl

These agents were tested for cross-reactions to the following human enteroviruses: polioviruses 1, 2 and 3; Coxsackie viruses A9 and B1-5; and ECHO viruses 1-14. Poliovirus antiserum pools of the three types did not neutralize two agents (No. 48 and No. 97), but did have some neutralizing effect on agent No. 93. Coxsackie rabbit antisera prepared by Microbiological Associates, Washington, D.C., USA, were capable of neutralizing the three prototype strains when used as a pool and, with the exception of the antisera prepared against Coxsackie A9 and B2, which did not neutralize No. 93, also neutralized all three prototypes when used individually. Luginbuhl (1958) considered this to indicate a widespread non-specific neutralizing capacity in rabbit antisera. Coxsackie monkey antisera at dilutions of 1:100 (5-100 units) did not neutralize any of the three prototype bovine viruses. Four pools of ECHO monkey antisera failed to bring about any neutralization of prototypes No. 48 and No. 97, and only one (containing types 1, 6, 9, 11 and 13) neutralized agent No. 93.

When sera from cattle were tested for neutralizing antibodies to human enteroviruses, a correlation between the prevalence of antibodies to polioviruses and the age of the animals was observed. Antibodies to type-2 virus were most prevalent and those to type 3 least prevalent. Acute and convalescent sera from animals harbouring agents were tested for evidence of increases in antibodies to human enteroviruses. Two out of four animals showed significant rises in antibody to poliovirus type 1 and in another group of four animals, one indicated an increase in antibody to poliovirus type 2. Four animal convalescent sera showed neutralizing substances to one ECHO and two Coxsackie viruses —namely, ECHO virus 2 and Coxsackie viruses B2 and B5.

This indication of poliovirus antibody in cattle has been noted by others: Hammon, Mack & Reeves (1947); Sabin & Fieldsteel (1955); Bartell & Klein (1955); and Klein (1958). In view of these

findings, Koprowski's isolation of type-1 poliovirus from the faeces of a calf may be even more noteworthy. During the period of this author's investigation, the animal in question responded with a low but significant increase (less than 1:2 to 1:8) in antibody to type-1 poliovirus. Klein (1958) found the frequency of neutralizing substances to the three polioviruses in approximately 150 cows to be 35%, type 1; 63%, type 2; and 11%, type 3. Neutralizing substances were also found to Coxsackie B viruses, types 4 and 5.

In order to ascertain whether or not these substances were true antibodies or non-specific inhibitors, Klein (1958) proposed that certain criteria be used as a guide. The suggested criteria for regarding a substance as an antibody were:

(*a*) heat stability at 60°C for 30 minutes;

(*b*) inactivation at 80°C for 20 minutes;

(*c*) presence in the globulin fraction;

(*d*) serological specificity, including animal variation, passive transfer, frequency increase with animal age, and booster effect of injection with the test organism;

(*e*) insensitivity to various chemicals used to eliminate non-specific inhibitors such as receptor-destroying enzyme, sodium periodate, trypsin, and fat solvents;

(*f*) failure to be removed by high-speed centrifugation;

(*g*) demonstration of its presence by several accepted tests: complement fixation, haemagglutination inhibition, neutralization, etc.;

(*h*) associated clinical and epidemiological data;

(*i*) isolation of the organism from the test animal.

It was pointed out that some of these criteria might not be applicable in certain cases. As a result of his investigations, Klein feels that the neutralizing substances described above were true antibodies.

The same question had been raised some ten years earlier by Hammon, Mack & Reeves (1947) in their studies with Lansing virus. Although these investigators found that some 70% of domestic animals contained " some type of antiviral substance ", they concluded that there were no grounds for assuming infection with Lansing poliovirus.

It may be coincidental, but the figure of 63% reported by Klein is in close agreement with that of 70% reported by Hammon and co-workers.

Enteric virus isolations from cattle have also been reported by Moll & Finlayson (1957). These isolations were from nine young calves (aged three to six months) which manifested temperatures of 104-106°F (40-41°C), slight cough, moderate discharge from the nose and large amounts of mucus in their stools. These animals maintained a normal food intake and recovered in a week without any therapy. Agents were isolated in bovine kidney cells from the faeces of four of the nine calves; from the faeces of two cows, one of which aborted; and from the ascites, stomach and placental fluids of the aborted cow. In another group of calves with the same clinical picture, the faeces yielded a similar virus agent. The CPE was rapid and dependent upon the TCD_{50}; for example, a low dilution of virus produced a CPE in four hours. Infectivity levels of $10^{12}TCD_{50}$ were obtained in replicate titrations. The virus was relatively stable, being ether-resistant and capable of storage for at least two months at -10°C to -22°C, for four days at 37°C, and for at least 30 minutes at 56°C. The pooled convalescent sera from four calves showed an increase in neutralizing antibody over the pooled acute sera. Intraperitoneal or intranasal inoculation of suckling or weaned mice did not produce signs of illness or pathological changes. Treatment of weaned mice with cortisone acetate (2-3 injections, 2.5-mg doses) increased their susceptibility to the virus so that extensive myocardial lesions were observed. Hsuing (1958a) reported that this bovine virus of Moll produced CP changes only in certain non-primate cell cultures.

Moscovici & Maisel (1958) employed rectal swabs to isolate 11 ECBO strains from over 100 " healthy " animals in rhesus monkey kidney cells. These investigators also demonstrated that their isolates could be separated into three groups on the basis of haemagglutination. Erythrocytes derived from man (type O), sheep, chickens, cows, guinea-pigs, and horses were tested with five strains agglutinating bovine cells and three different strains agglutinating guinea-pig cells when kept in the cold (5-8°C). This haemagglutination took one-and-a-half hours and did not occur at room temperature. It was also noted that rapid elution occurred and was completed within half an hour. Control viruses included 19 ECHO and 5 unclassified simian agents as well as bovine cells and non-inoculated monkey kidney tissue-culture fluids.

F. R. Abinanti (personal communication, 1958) recovered six or more enteroviruses from cattle

in bovine and monkey kidney tissue-culture. Studies with these agents are still limited, but one strain has been shown to be different from those described by Kunin. Several different viruses that on preliminary study appear to be enteroviruses have also been isolated from nasal swabs. These agents were associated with respiratory disease of cattle.

SWINE (ECSO) AGENTS

Moscovici, Ginevri & Mazzaracchio reported in 1956 the isolation of a CP virus from the stools of newborn swine. Some 20 suckling pigs with diarrhoea were studied, the isolate being obtained from a fatal case. The agent isolated in monkey kidney cells produced a similar CPE in pig kidney cells. No CPE was seen in rabbit kidney, human kidney, or HeLa cells. Chicken cells were not agglutinated at room temperature. Various routes of inoculation failed to produce infection in newborn pigs or in suckling or adult mice. Infection of two monkeys was achieved by oral administration of the virus, which was recoverable from the stools for 12 days. One of the two monkeys responded with a homologous antibody increase.

In studies to determine the prevalence of this agent in the swine population, rectal swabs obtained from adult pigs gave negative results. Serum studies on suckling pigs with and without diarrhoea failed to demonstrate antibody to this virus. Human gamma-globulin, anti-poliomyelitis monkey serum, and the antisera of many animal diseases (swine influenza, transmissible gastro-enteritis, porcine atrophic rhinitis, and canine distemper) failed to neutralize the agent. Neutralization of the virus was accomplished by sera from the sow of the litter containing the piglet that yielded the virus, from one pig recovering from an attack of hog cholera, and from six normal piglets. The agent did not cross-react with the swine virus isolated by Beran, Werder & Wenner (1958).

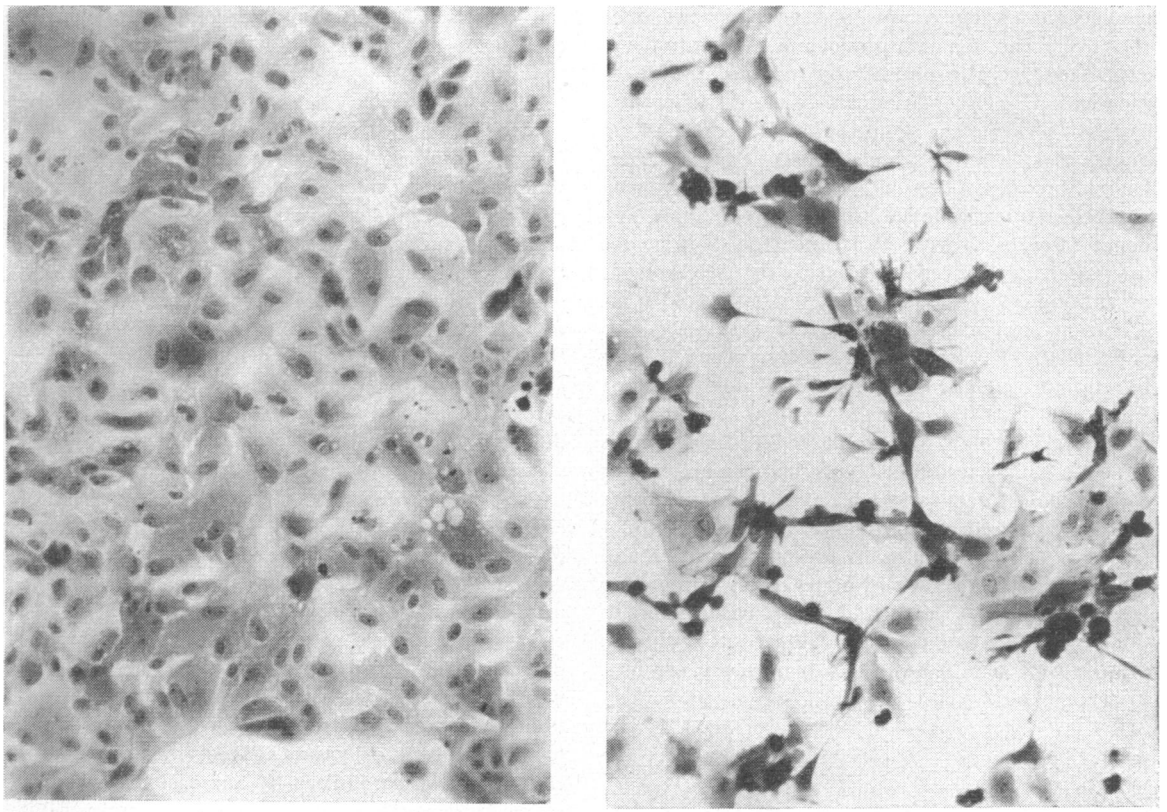
Beran and co-workers (1958) recently reported on their isolation studies with " healthy " swine. They obtained 376 CP agents in pig kidney-cell culture, using samples taken from the same animals at three different ages: 1-2 weeks, 5-6 weeks, and 9-10 weeks. The frequency of isolations increased, with increasing age, from 6.8% to 54.6% to 73.0%. Most of the isolates (121 out of 123) were antigenically similar. No relationship was shown between this strain and the following known viruses of man and animals: viruses of vesicular stomatitis, vesicular exanthema, swine influenza, hog cholera, transmissible gastro-enteritis, Teschen disease, atrophic rhinitis, and canine distemper; swine virus (Moscovici et al., 1956); swine virus (Dale & Songer, 1957); Newcastle disease virus; polioviruses 1, 2, and 3; Coxsackie viruses A9 and B 1-5; ECHO viruses 1-13; adenoviruses 1-8. Human and bovine gamma-globulins and three normal human sera failed to show evidence of neutralizing antibodies. On the other hand, pigs were widely infected, as evidenced by serological response and an 87.9% frequency of shed virus. This widespread infection is markedly different from the low frequency reported for the agent isolated by Moscovici, Ginevri & Mazzaracchio (1956). Additional studies by Beran (personal communication, 1958) provided evidence that the swine virus isolated was prevalent in northern and central Missouri. Serum samples from 29 herds, when assayed for neutralizing antibodies at a 1:4 dilution, showed evidence of neutralization in 27 of the 29 herds.

Titres were obtained in pig kidney cultures of approximately $10^{-6.5}TCD_{50}$ per 0.1 ml. Purification of virus was accomplished by the plaque technique of Dulbecco & Vogt (1954). Filtration through Seitz and 03 Selas filters did not result in loss of the agent. Although 30 minutes' exposure to ether did not destroy the virus, incubation at 56°C for five minutes did.

ECSO viruses were also isolated in pig kidney cultures by R. G. Webster (personal communication, 1958) in the routine examination of swine faeces. Five agents, all antigenically related and with little, if any, pathogenicity for pigs (piglets), rabbits, guinea-pigs, rats, or mice (suckling or adult), were studied. In addition to having a low virulence, these agents produced no CPE in HeLa, dog, or bovine kidney cells. Virus growth in pig kidney was rapid, with refractile cells noticeable in 12 hours and complete lysis apparent 48 hours after inoculation of 100 TCD_{50}. Fig. 3 shows the change from normal cells (A) to stellate cells (B) after 36 hours. No evidence of virus proliferation could be detected by various routes of inoculation of the chick embryo. The widespread prevalence of antibodies and passive transfer of antibody through colostrum suggest a similarity to the prototype strain described by Beran, Werder & Wenner (1958), but not to the strain described by Moscovici and his group (1956). There was no evidence of any serological cross-reactions with known viruses except for the neutralization of 10 TCD_{50} virus by type-3 poliovirus antiserum. The isolated viruses are probably true orphans as they

FIG. 3

CYTOPATHIC CHANGES IN PIG KIDNEY CELLS FOLLOWING INOCULATION OF 100 TCD_{50} OF ECSO VIRUS



A. Normal cells

B. Cells 36 hours after infection with 100 TCD_{50} of virus

Photograph supplied through the courtesy of Dr R. G. Webster

did not appear to be the cause of the sporadic deaths which occurred on farms from which the pigs were obtained. Virus was also isolated from kidney tissue-cultures prepared from experimentally inoculated pigs. Webster suggested that all enteroviruses can be isolated from kidney cells if tissue cultures are prepared while the animal is shedding virus.

CAT (ECCO) AGENTS

Fastier (1957) isolated an " orphan virus " from the spleen, lymph-nodes, and kidney of a cat dying of a laboratory-induced panleucopenia infection. No cytopathic changes were noted during the first two passages in kitten kidney epithelial cells. The third passage produced, on the third day, minor degenerative changes which became progressively more marked so that most cells were rounded and detached from the glass surface on the fifth day. After six transfers, the agent had a TCD_{50} of $10^{-6.8}$. The name "kidney cell-degenerating virus" or " KCD virus" was applied to the agent.

Growth-curve studies indicated that cytopathogenesis may be observed as early as ten hours after inoculation of 100 TCD_{50}. Maximal titres were obtained between 24 and 60 hours, depending upon the inoculum used. Cultures of several kitten tissues were able to support virus growth, but rarely to the titre obtained in kidney-cell preparations. Of interest was the elevated pH value (7.6-7.9) accompanying the autolysis of tissue fragments even after

continued incubation of cultures. The host range of this KCD virus was relatively limited. Only after intravenous inoculation did kittens manifest slight signs of illness: transient anorexia and diarrhoea, but no temperature changes. Neutralizing antibodies were produced by various routes of inoculation. Other animals and tissue-culture systems (suckling and young adult mice, one-day-old guinea-pigs, young rabbits, chick embryo liver, heart fibroblasts, dog kidney, and sheep embryo cutaneous muscle) failed to support or show evidence of virus growth. Virulence for the cat was low, infection being recognized by the development of antibodies.

Various physical and biological data were also collected by Fastier. Infectivity was retained after filtration through Seitz, EK, and Berkefeld W filters. The heat stability appeared limited, inactivation occurring after eight minutes at 56°C or four minutes at 60°C. Refrigeration (4°C) resulted in maintenance of infectivity for at least ten days, whereas freezing at −23°C caused a two-log drop in titre. Precipitation was accomplished with protamine sulfate and treatment with ether for 18 hours resulted in a drop from $10^{-7.2}$ to $10^{-5.4}$ in titre. No specific haemagglutinin could be demonstrated with group O human cells or with cells from guinea-pigs, sheep, rabbits, mice, or one-day-old chicks.

DISCUSSION

The use of tissue culture has resulted in the isolation of a number of agents from the excreta (primarily from stools) of man and animals. Many of these newly isolated viruses are not associated with specific diseases, and may be included therefore with viruses that are considered orphans. The relationship of these organisms to their host and to other orphans requires evaluation. In addition to complicating what may ordinarily be deemed a routine laboratory procedure, these new viruses have a biological significance to which consideration must be given.

In this review the major concern is the enteroviruses. However, brief mention must be made of the different animal tissues used for the development of tissue-culture cell lines. The presence of inherent tissue viruses, whether they be masked and inapparent or produce overt indications of infection, is capable of confusing the interpretation of data. It is obvious that not every tissue will continuously yield agents even though one may occasionally be recovered. Brown (1957) stated that rabbit kidney cells are all apparently free from "spontaneous occurring viruses". At present, however, the employment of many different animal tissues for cell lines is still limited and it would be premature to assume that the isolation of agents from these tissues does not or will not constitute a problem. Madin, Andriese & Darby (1957) reported on the successful cultivation in monolayers of various organs from cattle (kidney, testicle, cervix), dog (kidney), horse (kidney, tongue, neoplastic sarcoid), cat (kidney), adult and suckling guinea-pigs (kidney), adult and suckling hamsters (kidney, uterus), lamb (kidney, liver, testicle, brain), monkey (kidney, spleen), adult mouse (adenocarcinoma), suckling mouse (kidney, brain), adult and suckling rabbits (kidney), and swine (amnion, bladder, cornea, iris, kidney, liver, lung, ovary, sarcoma, testicle). Similar investigations and the widespread usage of these different tissues will undoubtedly reveal inherent viruses and incidentally demonstrate a susceptibility to still undiscovered agents. Hsuing & Melnick (1957) reported that kidney cells from different monkey species vary in their susceptibility to enterovirus and suggest that these cell lines offer a selective medium for isolation. Guerin & Guerin (1957) recommend the use of pig kidney-cell cultures for differentiating between Coxsackie and ECHO viruses, since such cells show a difference in susceptibility to these two groups of viruses. In contrast to this concept of tissue viruses, the problem of contamination was raised by Sabin (1957) when he questioned whether viruses isolated from various monkey tissues (and presumably this would apply to other animals) were really present in the tissues *per se* or were enteric viruses that contaminated the tissue during its removal from the animal body. Mention has been made of Webster's theory that enteric viruses can always be isolated from kidney cells so long as these cells were prepared at the time the animal was shedding virus. Although it is highly conceivable that contamination of a tissue by an enterovirus does occur, it is also significant that viruses have been detected in a particular tissue without their being demonstrated in faeces.

Little information is available regarding the isolation of orphan viruses from the alimentary tract of domestic and common laboratory animals such as horses, sheep, chickens, mice, rabbits, and hamsters. The isolation of lymphocytic choriomeningitis virus and Theiler's virus from mice has already been mentioned (see page 321). Luginbuhl and Burke (personal communication, 1958) have screened 103

poultry stool samples in chicken kidney cells and found CP agents in 42 specimens. Viruses have been isolated from domestic animals living in close proximity to man, although the intestinal flora of many animals, domestic and wild, is still to be determined. The reports cited here would suggest that the number of enteric cytopathogenic orphans still to be found must be relatively large.

These organisms are obviously global in distribution. Similarities among strains isolated in different countries have already been demonstrated—for example, among ECBO viruses in central USA and Northern Ireland, and among ECSO viruses in the USA and New Zealand. Strain differences must also be expected from various geographical areas. Certain isolates will be reclassified and not considered orphans as their association with disease processes is demonstrated. The discovery by Lee & Gillespie (1957) of an agent responsible for virus diarrhoea of cattle is in keeping with this observation.

The occurrence in animals of enteroviruses that are counterparts of those found in humans prompts speculation regarding the existence of some common relationship between the many agents of different species and the possibility that the characteristic growth or disease pattern of the virus occurs only when it is placed in its preferential host cell or tissue. In this regard, the findings of Verlinde, Versteeg & Beeuwkes (1958) are extremely important. During an outbreak of respiratory disease among children, a Coxsackie A, type-5 virus was isolated repeatedly from the lungs of two pigs. Convalescent sera from the children all showed high neutralization indices to this virus. Hull & Minner (1957) report that SV 22 (supplied by Dr R. J. Heubner) contained an adenovirus complement-fixing antigen. Other simian viruses (CPE group 1) were also found to produce complement-fixing antigens in common with adenoviruses, along with other characteristics (Hull, Minner & Mascoli, 1958). In addition, the suggestion was made that " rhesus and cynomolgus monkeys may carry groups of viruses which are the simian counterparts of human adenoviruses, Coxsackie, ECHO, herpes and possibly other groups of viruses ". One virus, SV 5 (CPE group 4), was shown to be serologically related to the mumps virus and similar to the croup virus isolated by Chanock (1956). This resemblance of monkey virus isolates to human viruses was also noted by Hoffert, Bates & Cheever (1958).

After comparing ECMO (SV 23, 16, and 6), ECBO (Moll & Finlayson, 1957) and ECHO 10 viruses in primate (rhesus, *patas*, capuchin) and non-primate (swine, cat, dog, calf, rabbit, guinea-pig) tissue-cultures and, in addition, studying plaque formation in rhesus monolayers, Hsuing (1958b) observed that ECHO 10 virus behaved differently from other enteroviruses. Certain of the tested animal viruses, however, possessed the same growth characteristics as the ECHO 10 virus. Prier & LeBeau (1958) studied the cytological changes induced by several simian viruses and adenoviruses in monkey kidney cells and noted that one simian virus (SV 27) produced alterations in the cell nucleus that were indistinguishable from those manifested by adenoviruses types 3 and 4. Several investigators have suggested the possible relationship between the virus of distemper and that of measles (Adams, 1953; Carlström, 1957). Koprowski (1958) cites the studies of Polding and Simpson, who report a possible relationship between the rinderpest and the distemper virus. Koprowski's jesting supposition that " perhaps one day children will be vaccinated with distemper virus against measles, dogs with rinderpest virus against distemper, and cattle with measles virus against rinderpest " is indeed intriguing.

There is sufficient documentation to indicate the existence of many viruses in nature in an inapparent or masked form. Previous studies with a number of viruses have established the concept that this phenomenon occurs in one or another form. The early studies with influenza virus are well documented (Henle, 1953). The virus diarrhoea agent of Lee & Gillespie (1957) produces very little, if any, CPE, although good virus growth occurs in the host cell. The investigations of Rowe and co-workers on adenoviruses (Rowe et al., 1953) and human salivary-gland virus (Rowe et al., 1956) in adenoids indicate invasion of natural hosts without clinical manifestations. Hotchin & Cinits (1958) were able to induce in suckling mice an indefinite carrier state without detectable neutralizing antibodies, with no inflammation, and with no capacity to produce a CPE *in vitro* with inoculations of lymphocytic choriomeningitis virus that produced infections in older animals.

This problem of latency and masked virus has recently been reviewed by Ginsberg (1958), who emphasized the lack of information concerning the actual mechanism of the carrier state in that set of circumstances whereby undetected virus persists within the host tissue for long periods of time. Ginsberg also indicated that the carrier state in tissue culture may have a similar mechanism of infection to that in nature, thus offering the oppor-

tunity for investigations into the persistence of viruses within the host cell. One factor common to this carrier state, and pertinent to this discussion, is the resistance of host cells to injurious activities of virus.

Is it possible, then, that these data support the hypothesis that the basic transmissible particle is an incomplete form of infectious nucleic acid? This nucleic acid maintains in nature the infectiousness of the virus and provides the molecular pattern for genetic control as well as stimulation for the development of the protein moiety necessary for specific antigenicity. This would then explain the ability of a virus to cause inapparent infection as well as its ability to exhibit antigenic characteristics common to several different viruses when in one particular host.

The continued recording of the presence of poliovirus-neutralizing substances in animal sera also lends credence to this concept. The studies cited here not only agree concerning anti-poliovirus substances, they have also contributed to the problem by demonstrating the presence of antibodies to Coxsackie and ECHO viruses in a number of animal sera.

Many investigators have now reported the presence of anti-poliovirus substances in the sera of animals under study. The pioneer studies by Flexner & Lewis (1910) alluded to neutralizing substances for polioviruses in sheep serum. Gordon (1945) found that a small number of dog sera (3 out of 37) contained neutralizing antibodies to poliovirus type 2 (Lansing). As mentioned earlier, Hammon, Mack & Reeves (1947) showed that 70% of domestic cows and horses had " some type of antiviral substance ". Similar test animals from isolated areas did not show, or only infrequently showed, the presence of an anti-poliovirus-2 (Lansing) substance. The last-mentioned investigators also found that the sera from 44% of unprotected chickens, as compared to 4.6% of laboratory chickens, were able to protect mice against this same virus. Because poliovirus inoculations of chickens by various routes failed to produce antibody, it was felt that these chicken anti-poliovirus substances were non-specific. It was, however, concluded that the presence of these substances in domestic animals was a result of the animals' living in close proximity to man. More recently, Gear (1952) demonstrated the presence of anti-poliovirus-2 substances in sera obtained from cows, chickens, ducks and pigeons. Evidence that these neutralizing substances were antibodies needs further study. While it is true that Klein suggests that these substances have the characteristic of antibody, he also poses the question of their origin. Sabin & Fieldsteel (1955) failed to induce poliovirus infection in calves, but they demonstrated that the neutralizing substances in calf serum had similar properties to human serum antibody. The suggestion was made that this response resulted from infection by an agent or agents antigenically related to poliovirus. McFerran (personal communication, 1958), while studying ECBO viruses, noted that a number of cattle developed a neutralizing substance to poliovirus (type not indicated). Studies are still in progress regarding the reason or reasons for this development. The isolation by Koprowski of poliovirus type 1 from calf stools has already been mentioned. Confirmation of this would not only be of immense importance, but would also magnify the need for additional large-scale isolation studies among animals that are closely associated with man.

A hypothesis concerning the exact nature of virus particles as they exist in nature has already been discussed. One would expect, as part of this hypothesis, the occurrence of virus mutants. Enders, Weller & Robbins (1952) and others recognized the existence of poliovirus mutants. Takemori and his co-workers (1957a, 1957b) were able to produce resistant poliovirus mutants by cultivating the virus in the presence of inhibitors found in normal bovine serum.

Comparison of kinetic curves of inactivation of mutants by normal bovine inhibitor and homologous antiserum led to the suggestion that this neutralizing substance was not antibody (Takemori et al., 1958). However, the antigenic stimulation of these presumed normal animals prior to their arrival at the abattoir was unknown—a fact that provoked the question of the origin of these substances. It would also be of interest to ascertain whether these substances and those previously reported by Hammon, Mack & Reeves (1947) are similar. The recent finding of Oudin (1956) that antigen-antibody reaction patterns are varied implies additional studies on the nature of this immunological response.

The emergence of these enteroviruses will pose numerous problems to investigators. The existing unknown status of many of these agents will be complicated by an increased activity and interest in this field. Detailed studies will become increasingly difficult as more and more agents are reported. This

enhanced activity will necessitate expanded comparisons of new strains with those previously described. This may be exemplified by two recent and separate reports. Gelfand (1958) described two strains of " ECDO " virus isolated from healthy stray dogs in dog kidney-cell cultures, but a more detailed report of this study failed to substantiate the original observation (Gelfand & Flynn, 1958). The CP agents originally isolated proved to be the virus of infectious canine hepatitis. Thus, to date, no true ECDO viruses have been described. It may be of interest to note that Gelfand and Flynn failed to find any evidence of antibodies to human enteroviruses in the sera of the dogs studied.

Reference sera and prototypic viruses will be essential for the successful completion of classification and for interrelationship studies. This would suggest the need for a central source of typing sera and prototypic strains in order to prevent confusion. A strain-typing centre, perhaps similar to that available for influenza, would be of value for the enteroviruses. This centre would function not only as a typing and classification centre, but also as a source of information.

Without too much difficulty one can foresee the isolation and description of such virus families as ECCO (cat), ECFO (fish), ECAO (bird, *avis*), ECEO (equine), ECRO (rodent), etc. Some concern has been expressed over the use of these terms for grouping viruses. This is based on the assumption that confusion will occur as more and more viruses are isolated and described. Confusion need not develop in classifying and integrating these new isolates. A new agent from a particular animal should first be tested against organisms previously isolated from the same host. Continued studies would indicate relationships to other agents isolated under similar circumstances, perhaps from other hosts. If no disease process was associated with the isolate or could be experimentally induced, the " orphan " status becomes important. Therefore until the interrelationships are determined the present system indicates the original host of the agent and the lack of relationship to previously described viruses.

One source of difficulty that may ensue from assigning family names is exemplified by the facetious assignment of the term " ECEO " to agents that may be isolated from elephants (Kalter, unpublished data). It is now suggested that this term be reassigned to horses merely because of the greater probability of studying horses than elephants. However, if perchance an agent is isolated from elephants, little is left but to name it " ECPO " (pachyderm).

Difficulties often occur following the introduction of different classification systems by various investigators. It would appear, if we are to follow precedent established in naming the ECHO viruses, that a simple numerical sequence for each isolate would be sufficient. Thus, the ECMO viruses would be ECMO 1, 2, 3, 4, etc., the swine viruses would be ECSO 1, 2, 3, 4, etc. The ECMO viruses have also been grouped by different investigators according to their ability to produce a characteristic CPE (Hull & Minner, 1957) or according to their plaque formation on rhesus and *patas* monkey kidney cells (Hsuing & Melnick, 1958). As there are similarities between the two systems, perhaps one system should be established incorporating the salient features of both.

In conclusion, there are many considerations needing emphasis or perhaps re-emphasis. Numerous animals are yet to be studied. The primary objective of this review was to describe the newly isolated animal viruses and postulate that agents from both domestic and wild animals will continue to be discovered. The possible host range of many of " man's best friends " to viruses that are infectious for man needs extensive examination. It would appear that so long as animal tissues serve as a source of culture, whether as a host system or in a primary investigation, inherent viruses will be a potential source of contamination. Intestinal viruses causing a number of disorders exist in man and animals. Our lack of information regarding diarrhoea, constipation, enteritis, synergistic reactivities, etc. indicates the enormity of the field. Agents have been, and most certainly will be, isolated from specific clinical conditions. The laboratory procedures, mainly serological, required to correlate these vast numbers of agents to other known or yet-to-be-discovered viruses are almost astronomical in scope. The organization and integration of these agents will constitute a major effort by a number of investigators. This review indicates the present lack of information.

RÉSUMÉ

La découverte du pouvoir cytopathogène des virus pour les cellules en culture et la méthode d'isolement sur culture de tissu qui en a résulté ont conduit rapidement au dépistage d'un nombre considérable de virus dans le tractus digestif de l'homme et des animaux. Certains de ces virus avaient été prélevés sur des sujets affectés de maladies apparentées à la poliomyélite, mais d'autres provenaient de sujets apparemment sains. On a appelé « orphelins » ces virus que l'on ne peut mettre en relation avec aucun syndrome morbide reconnu. Les virus intestinaux trouvés chez l'homme ont été désignés comme appartenant au groupe ECHO (entero-cytopathogenic, human, orphan). On rencontre chez l'animal des virus homologues: chez le singe (ECMO), chez les bovidés (ECBO), chez le chat (ECCO), ce qui pose la question des relations entre virus humains et animaux.

L'auteur passe en revue les connaissances actuelles sur ces virus. Il expose la difficulté de leur identification sur des tissus qui peuvent eux-mêmes être spontanément infectés par des virus inapparents. La question des virus « masqués », de l'infection latente, est ensuite abordée. Leur existence est maintenant établie, mais on ignore sous quelle forme le virus peut persister, souvent de longues périodes, chez son hôte sans y déclencher aucun symptome clinique. L'hypothèse d'une forme incomplète de l'acide nucléique infectant est actuellement celle qui rend le mieux compte des faits observés. La présence de substances antipoliomyélitiques dans le sérum de certains animaux, ainsi que celle d'anticorps anti-ECHO et anti-Coxsackie semble venir à l'appui de cette théorie et ouvrir de nouvelles voies à la recherche, chez l'animal, des virus pathogènes pour l'homme.

L'essor que les recherches ont pris ces dernières années, va probablement s'intensifier encore. Si l'on veut éviter la confusion que créera sans doute la description et la caractérisation sérologique de centaines de ces virus, il faut songer à l'établissement de sérums de référence et de souches prototypes, auxquels comparer les virus nouvellement découverts. L'auteur souligne les services que rendrait un centre de référence et d'information analogue à celui qui existe pour la grippe et d'autres maladies à virus.

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