Poliovirus Mutants with Altered Responses to Cystine

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SUMMARY: Three strains of poliovirus (Akron, antigenic type 1; Brooks, type 2; Mabie, type 3), which require exogenously supplied cystine (or a cystine-substitute) for optimal cytopathogenic action on monkey kidney tissue cultures, were serially passaged ten times in cystine-deficient monkey kidney tissue cultures in tubes. From various passage levels in this system were isolated three mutants of Akron, four of Brooks and two of Mabie. These nine mutants are designated the *cystine-response* mutants and are distinguishable from their ancestral viruses used to initiate the passage series by being relatively cystine-non-requiring. Two criteria suffice to distinguish the mutants of any strain from each other: (1) the degree of loss of requirement for cystine, and (2) the degree of inhibition by relatively high concentrations of cystine. The compositions of the virus populations in most of the passage levels were determined, and from these determinations the patterns of emergence of the mutant viruses were delineated.

Several poliovirus mutants which are classifiable on mammalian cells *in vitro* have been obtained. Dulbecco & Vogt (1955) reported an *r* (*rapid*) mutant of the Brunhilde strain (antigenic type 1). This *r* mutant was originally characterized by its relatively large plaques on monkey kidney tissue cultures in a medium containing embryo extract but has since been renamed the *d* (*delayed*) mutant because it can be more readily classified on the basis of its characteristic delay on monkey kidney cells maintained in a relatively acid medium (Vogt, Dulbecco & Wenner, 1957). Several independent occurrences of this type of mutant in other strains of poliovirus have been reported (Sabin, 1956; Vogt *et al.* 1957). A heat-resistant variant of the MEF1 (type 2) strain of poliovirus was obtained by Stanley, Dorman, Ponsford & Larkin (1956) and heat-resistant mutants (t_1 and $t_1 t_2$) of the Brunhilde strain were reported by Dulbecco (1956). Slow Mahoney (Dubes, 1956*a*) is an *m* (*minute*) mutant of the Mahoney strain (type 1) and can be readily classified on the basis of its tiny plaques on monkey kidney tissue cultures. Cold-adapted genetic variants (Dubes & Chapin, 1956) of strains Akron (type 1), Brooks (type 2) and Mabie (type 3) are characterized by their relatively high rate of propagation on monkey kidney tissue cultures maintained at 30°. Genetic variants further adapted to the HeLa cell system have also been obtained (Dubes & Wenner, 1957). Recently, Takemori *et al.* (1957) reported Mahoney and MEF1 mutants which are resistant to the substance(s) which can be found in normal adult bovine sera and which inhibits the 'wild-type' Mahoney and MEF1 viruses.

The demonstration that three strains (Akron, Brooks, Mabie) of poliovirus require exogenously supplied cystine or a cystine-substitute for optimal cytopathogenic action on monkey kidney tissue cultures (Dubes, 1956*b*) and the

general success of the technique of passage under conditions favouring the selection of mutants suggested the possibility of obtaining cystine-non-requiring mutants through passage of polioviruses on cystine-deficient monkey kidney tissue cultures. The emergence and characterization of relatively cystine-non-requiring mutants obtained through such selective passages are the subject of this paper. Part of this work has been reported in abstract form (Dubes & Chapin, 1957). The relative virulence in monkeys of some of these mutants has been studied and will be reported elsewhere (Dubes & Wenner, 1957), with a brief summary of their characterization on cells *in vitro*.

METHODS

Poliovirus strains. Akron purified pool no. 1 (PP1), Brooks PP1 and Mabie PP1a, all of which are cystine-requiring strains, were chosen for passage and study. The formation of these purified pools has already been described (Dubes, 1956a).

Monkey kidney tissue cultures. Kidney cells from rhesus (*Macaca mulatta*) or cynomolgus (*M. irus*) monkeys were dispersed with trypsin and grown on medium D (Younger, 1954) for 6 days at 35–37° in 150 × 16 mm. test tubes, 60 mm. diameter Petri plates, or 200 ml. screw-cap serum bottles with square cross-section. In a few experiments the growth medium of Melnick (1955) was used in place of medium D. After this growth period the cultures were washed three times with cold phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), the tube cultures each time with 2.0 ml., the plate cultures each time with 4.25 ml., and the bottle cultures each time with 10 ml. After virus inoculation, the cultures were maintained at 35–37° on 0.90, 6.0 and 10 ml. of a maintenance medium for tubes, plates and bottles, respectively. The maintenance overlay for the Petri plates contained 0.90% (w/v) washed agar. The maintenance media used were based on Eagle's maintenance medium (E_m) (Dubes, 1956b). Medium E_m and an altered E_m (AE_m), which was developed by Miss Joanne Rush for use in this laboratory, were used without cystine, or with cystine at concentrations within the range of 0.050–1.0 mM. Medium AE_m differs from E_m in that it contains Hanks's balanced salt solution (Hanks & Wallace, 1949) in place of the salt solution used by Eagle (1955); the concentration of sodium bicarbonate is, however, kept at 20 mM. All maintenance media were adjusted to a pH value between 6.4 and 6.8 for use in tube and bottle cultures, and to pH 7.0 for use in the plaque method on Petri plates. Tubes and bottles were sealed. Cultures in tubes were rolled after inoculation. Petri plate cultures were kept in a humidified incubator which was continuously flushed with 3% (v/v) carbon dioxide and 97% (v/v) air (Dulbecco & Vogt, 1954) and were stained with neutral red 4–8 hr. before examination (Dubes, 1956a). In general, the plaque method used was adapted from the original method of Dulbecco & Vogt (1954).

Inoculations with viruses. Viruses were diluted in PBS; 0.10, 0.30 and 1.0 ml. of each appropriate dilution were inoculated into tube, plate and bottle cultures, respectively.

Storage of viruses. All virus stocks were stored in rubber-stoppered ampoules in a dry-ice box.

Passages of viruses on kidney cultures unsupplied with cystine. Samples from Akron PP1, Brooks PP1 and Mabie PP1a were passaged ten times serially in tissue cultures maintained on E_n without cystine. The inoculum for each passage was virus usually at 10⁻² concentration, equivalent to 10³ to 10⁵ plaque-producing particles inoculated per tube. Between two and six tubes were used for each passage of each strain. Tube cultures were harvested when they exhibited 90–100 % cytopathogenic degeneration. The tenth-passage virus of each strain was then passaged three times serially by the plaque method on cells maintained on E_m minus cystine. Each third serial plaque isolate was then inoculated for pool formation into several bottle cultures on the same medium. The pools so obtained were designated Akron PP5, Brooks PP5 and Mabie PP5. The virulence in monkeys of these three viruses has been studied (Dubes & Wenner, 1957).

Genetic purification and pool formation with the other viruses emerging during the passages. From various of the tube-passage levels polioviruses differing from both the PP1's and PP5's were isolated. Purifications and pool formations with these viruses were performed in the same manner as with the PP5's, except that in some cases the purifications by the plaque method were performed on cystine-supplied cells so that plaques of desired appearance might be selected for isolation.

Typing of viruses. The antigenic type of each virus pool was confirmed in neutralization experiments against the three specific poliovirus antisera formed in monkeys (Wenner, Miller, Kamitsuka & Wilson, 1954) and against normal monkey serum as a control.

Symbols for the mutant viruses. To avoid unwieldy descriptions when referring to the various mutants the designations and symbols defined in Table 1 have been used. These symbols do not imply that the phenotypic differences between the various mutants are due to single genetic differences. Akron PP1, Brooks PP1 and Mabie PP1a, the cystine-requiring viruses from which the mutants were derived, are referred to as wild-type (+^{cr}) viruses in regard to cystine response. Akron PP5 and Mabie PP5 are *crⁱ* viruses, whereas Brooks PP5 is *cr* virus. The criterion of degree of inhibition by cystine (Table 1) is based on the 'turbidity' of plaques produced on cells supplied with a relatively high concentration (0.20–1.0 mM) of L-cystine. 'Turbid' plaques are plaques which contain a relatively large fraction of neutral red-stainable cells.

Environmental factors affecting the cystine response. The manifestation of a requirement for cystine by the cystine-requiring polioviruses can be modified by varying the temperature during viral action or by establishing the kidney cultures on a different growth medium. For these viruses a lowering of the temperature from 37° increases the relative plating efficiency on cells not supplied with cystine. Substituting the growth medium of Melnick (1955) for medium D has a similar effect. These modifications may operate through altering the size of the intracellular pool of cystine or 'cystine-activity', the

influence of the composition of the growth medium being on the size of this pool at the time of inoculation and the influence of temperature being on the rate of metabolic change of this pool after inoculation.

Table 1. *Designations and symbols for wild-type and cystine-response (cr) mutant polioviruses*

+ + = clear requirement for, or strong inhibition by, cystine. + = slight requirement for, or slight inhibition by, cystine. 0 = no demonstrated requirement for, or no demonstrated inhibition by, cystine.

Virus designation	Virus symbol	Response to cystine	
		Requirement for	Inhibition by
Wild-type	+ ^{cr}	+ +	0
Cystine-response—partial	cr ^p	+	0
Cystine-response	cr	0	0
Cystine-response—slightly inhibited	cr ^{si}	0	+
Cystine-response—inhibited	cr ⁱ	0	+ +

RESULTS

Akron cr mutants

Early tests demonstrated clearly that Akron PP5 (crⁱ) differs from its progenitor PP1 (+^{cr}) virus in response to cystine. The Akron crⁱ mutant shows no requirement for exogenously supplied cystine under the conditions employed: moreover, it produces 'turbid' plaques on monkey kidney cells under an agar layer which contains a relatively high concentration (0.20 to 1.0 mM) of L-cystine.

Platings of the various tube-passage virus populations leading to the isolation of the crⁱ mutant were made on cells unsupplied and on cells supplied with cystine. In these experiments the +^{cr} and crⁱ viruses were similarly plated so that comparisons could be made with the viruses at different passage levels using three criteria: (1) plating efficiency without cystine relative to efficiency with cystine; (2) plaque size with and without cystine; and (3) plaque appearance with and without cystine. The results obtained from these comparisons indicated that two other viruses, the cr and cr^{si} mutants, which are different from each other and from both the +^{cr} and the crⁱ viruses, were also obtained in the series of passages on cystine-deficient cells in tubes. Purified pools of the cr and cr^{si} mutants were prepared from isolates obtained from tube-passage levels 2 and 5, respectively. The cr mutant may be characterized as cystine-non-requiring and cystine-uninhibited and the cr^{si} mutant as cystine-non-requiring and slightly cystine-inhibited. The data from a typical experiment showing the differentiation of these three Akron *cystine-response* mutants and of wild-type Akron are presented in Table 2. In this experiment the cells had been grown on medium D and plaques developed for 4.0 days in a continuously flushed incubator at 36.6°. The difference between the +^{cr} and crⁱ viruses is illustrated by Pl. 1, fig. 1.

The proportions of the +^{cr}, cr, cr^{si} and crⁱ Akron polioviruses in the various tube-passage levels were estimated by using the three criteria mentioned above

(relative plating efficiency, plaque size and plaque appearance) and by characterization of the viruses in some plaque isolates from some passage levels by the same criteria. Such classifications of the viruses in the passage levels gave the pattern of emergence of these three Akron mutants presented in Fig. 1.

Table 2. *The differentiating characteristics of the wild-type and cystine-response mutant Akron, Brooks and Mabie polioviruses on monkey kidney cells in vitro*

	RPE (%)	Differentiating characteristics			
		Mean plaque diameter (mm.)		Plaque appearance	
		With cystine*	Without cystine†	With cystine*	Without cystine†
Poliovirus					
Akron + ^{cr}	0.14	3.7	1.6	Clear	Clear
Akron <i>cr</i>	71	4.4	4.3	Clear	Clear
Akron <i>cr^{si}</i>	190	2.5	6.0	Slightly turbid	Clear
Akron <i>crⁱ</i>	130	3.9	5.6	Turbid	Clear
Brooks + ^{cr}	0.069	7.1	2.9	Clear	Clear
Brooks <i>cr^p</i>	57	7.8	4.0	Clear	Clear
Brooks <i>crⁱ</i>	120	5.4	3.4	Turbid	Slightly turbid
Brooks <i>cr^{si}</i>	110	5.7	5.9	Slightly turbid	Clear
Brooks <i>cr</i>	200	6.4	6.0	Clear	Clear
Mabie + ^{cr}	2.1	7.0	1.2	Clear	Clear
Mabie <i>cr^{si}</i>	120	4.8	4.5	Slightly turbid	Clear
Mabie <i>crⁱ</i>	260	3.5	4.8	Turbid	Clear

* Medium AEm with L-cystine at 1.0 mm for Akron and Brooks and at 0.50 mm for Mabie.

† Medium AEm without cystine. RPE (relative plating efficiency) is the plating efficiency without cystine relative to the efficiency with cystine. The data are from 3 experiments, 1 for each strain. See Table 1 for an explanation of the symbols for the viruses.

Brooks cr mutants

The general procedure followed with Akron was also used with Brooks. The virus in Brooks PP 5 produces clear plaques in the presence of high concentrations of cystine, in contrast to the turbid plaques produced by Akron PP 5, and is thus designated *cr*. This *cr* mutant may be differentiated from the original Brooks +^{cr} virus on the basis of its lack of requirement for exogenously supplied cystine. Comparisons of the tube-passage virus populations with the +^{cr} and *cr* viruses indicated that three other mutants, the *cr^p*, *crⁱ* and *cr^{si}* mutants, also emerged during the series of tube passages. Purified pools of the *cr^p*, *crⁱ* and *cr^{si}* mutants were prepared from isolates obtained from tube-passage levels 1, 2 and 4, respectively. In Table 2 data from a typical experiment showing the distinguishing characteristics of these three mutants and the +^{cr} and *cr* viruses are presented. In this experiment the cells had been grown on medium D and the plaques developed for 4.0 days in a continuously flushed incubator at 35.8°.

The pattern of emergence of these four Brooks *cystine-response* mutants was determined in the same way as was the pattern for Akron and is shown in Fig. 1.

Fig. 1. The patterns of emergence of the *cystine-response* mutants. PP1 (PP1a for Mabie) is the starting pool of $+^{cr}$ virus. Designations P1 through P10 indicate the serial numbers of rapid consecutive passages in tube culture. See Table 1 for an explanation of the symbols for the viruses. Data for P8 and P9 of Akron and for P6 of Mabie were not obtained.

Mabie cr mutants

Mabie PP5 contains a virus which has a cystine-response similar to that of the Akron cr^i mutant and is thus called the Mabie cr^i mutant. Another Mabie mutant, the cr^{si} mutant, also emerged during the tube passages. A purified pool of this mutant was prepared from an isolate from tube-passage level 5. Data which show the differentiation of the Mabie cr^{si} , cr^i and $+^{cr}$ viruses are given in Table 2. These data were taken from a typical experiment in which the cells had grown on medium D and the plaques developed for 4.0 days in a continuously flushed incubator at 36.7°. A visual comparison of the $+^{cr}$ and cr^i viruses is afforded by Pl. 1, fig. 2.

The pattern of emergence of these two Mabie *cystine-response* mutants was determined and is shown in Fig. 1.

Tests of progeny viruses in plaques formed by $+^{cr}$ viruses on cells not supplied with cystine

Since viruses with altered responses to cystine appeared so very early in the passage series (Fig. 1) experiments were done to see if a single-plaque passage

of a $+^{cr}$ virus on cystine-deficient cells would give a virus with an altered cystine-response. Nine, 7 and 3 plaques formed by Akron, Brooks and Mabie $+^{cr}$ viruses, respectively, on cystine-deficient cells were isolated and the cystine-responses of the progeny viruses therein were determined. Three of the Akron plaque isolates were characterized in tests performed in tube cultures, a method previously described (Dubes, 1956*b*). By these tests the three isolates were not unlike their Akron $+^{cr}$ parent. The other 6 Akron isolates were studied more precisely by the plaque method; one was found to be similar to the Akron cr virus and the other 5 were found to be intermediate between $+^{cr}$ and cr . Two of the latter group of 5 isolates were further resolved by characterization of plaque subisolates. From one of these a virus of phenotype intermediate between $+^{cr}$ and cr and a virus somewhat similar to but probably different from cr^{si} were isolated. From the other isolate the two subisolates tested proved to be $+^{cr}$. Three of the 7 Brooks isolates were studied in tests in tube cultures and not found to be different from Brooks $+^{cr}$ virus. The other 4 Brooks isolates were studied by the plaque method; 2 were found to be like the Brooks cr^p virus and 2 were found to be intermediate between the $+^{cr}$ and cr^p viruses. From one of these 'intermediate' isolates 5 subisolates were obtained. Three of these 5 subisolates proved to be like the original 'intermediate' isolate, one was found to be similar to the Brooks cr^i virus, and one was $+^{cr}$ virus. The three Mabie isolates were tested in tube cultures and not found to differ from their progenitor virus, Mabie $+^{cr}$. No tests of Mabie isolates by the plaque method were made since the results of these tests in tube cultures and the finding that tube-passage level 1 contained $+^{cr}$ virus (Fig. 1) show that *cystine-response* mutants of Mabie do not *necessarily* appear after propagation of Mabie $+^{cr}$ virus on cystine-deficient cells.

Thus relatively cystine-non-requiring viruses of strains Akron and Brooks may be obtained by the isolation of plaques formed after the inoculation of the $+^{cr}$ viruses on to cystine-deficient cells. The data also indicate that some of the progeny viruses in these plaques have an unaltered cystine-response.

Tests of progeny viruses in plaques formed by the Akron cr^i , Brooks cr and Mabie cr^i viruses (PP5's) on cystine-supplied cells

Plaques formed by Akron cr^i , Brooks cr and Mabie cr^i viruses on cystine-supplied cells were examined for the presence of viruses with cystine-responses different from those of their parents. Such viruses might possibly arise from genetic reversions or simply from the fact that they were synthesized in cystine-supplied cells (cf. Luria, 1953). Seven isolates from typical Akron cr^i plaques were tested and found to contain cr^i virus. An isolation was also made from an atypical Akron cr^i plaque. This plaque was atypical in that it contained a relatively clear sector, i.e. a region in which a relatively low fraction of the cells had concentrated the neutral red. Such atypical sectorized plaques are not infrequently found when Akron cr^i virus is plated on cells supplied with cystine at 0.20–1.0 mM. From the relatively clear sector of this plaque was isolated a virus unlike cr^i but somewhat similar to $+^{cr}$. It differed, however, from $+^{cr}$ in producing slightly turbid instead of clear plaques on

cystine-supplied cells and possibly also in having an even lower relative plating efficiency on cells not supplied with cystine. Eight and 6 typical plaques from Brooks *cr* and Mabie *crⁱ*, respectively, were isolated. Each contained virus like its parent.

These findings show that the progeny viruses in typical plaques formed by the cystine-non-requiring Akron *crⁱ*, Brooks *cr* and Mabie *crⁱ* viruses on cystine-supplied cells are like their parental viruses. Thus the nature of these progeny viruses is not determined by the amount of cystine supplied to the cells in which they were synthesized. The cystine-requiring virus which was obtained from the sector of a sectored plaque formed by Akron *crⁱ* virus very probably arose through genetic reversion from *crⁱ* during the propagation of the *crⁱ* virus on the cystine-supplied cells.

DISCUSSION

The patterns of emergence of the relatively cystine-non-requiring viruses during the passages in tube cultures and the isolations of +*cr* viruses from the progeny viruses in some of the plaques formed by the +*cr* viruses on cells not supplied with cystine indicate that the variant viruses were obtained through the processes of mutation and selection. The ease of obtaining relatively cystine-non-requiring mutants, of the Akron and Brooks strains especially, is emphasized by the fact that the characterization by the plaque method of the progeny in 6 plaques formed by Akron +*cr* virus and of 4 plaques formed by Brooks +*cr* virus on cells not supplied with cystine revealed the presence of at least three different kinds of *cystine-response* mutants of Akron and at least three of Brooks. These results suggest that the formation of most of the plaques produced by Akron +*cr* and Brooks +*cr* on cystine-deficient cells under the conditions usually employed herein is in good part due to mutation from +*cr* to various relatively cystine-non-requiring mutants during the limited propagation of the +*cr* viruses on the plate cultures.

The isolation from a relatively clear sector of an otherwise turbid plaque formed by Akron *crⁱ* virus on cystine-supplied cells of a virus genetically reverted from *crⁱ* indicates the genetic lability of Akron *crⁱ* virus. It is interesting to note that the cystine-non-requiring characteristic of Akron *crⁱ* virus is apparently completely revertible in one mutational step. The ease of locating the mutant sectors in this system suggests that it may be useful for a study of the process of mutation in polioviruses.

The success of these passages of polioviruses on cystine-deficient monkey kidney tissue cultures in obtaining *cystine-response* mutants and the analogous success in obtaining cold-adapted genetic variants (Dubes & Chapin, 1956) indicate the general usefulness of the technique of passage on cells under abnormal conditions. Thus one kind of host cell may be utilized to obtain different classes of mutant viruses simply by altering the physicochemical environment for the cell-virus system in different ways.

The de-adaptation to the original host cell system (cystine-supplied cells) shown by the Akron and Mabie *crⁱ* viruses is analogous to the de-adaptation to

cells at 37° shown by the cold-adapted genetic variants of Akron, Brooks and Mabie (Dubes & Chapin, 1956; Dubes & Wenner, 1957) and reminiscent of the classic de-adaptation to the original animal host observed in animal viruses after serial passage in a different animal host (e.g., Pasteur, Chamberland & Roux, 1884).

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Fig. 1

Fig. 2

G. R. DUBES AND M. CHAPIN—CYSTINE-RESPONSE MUTANTS OF POLIOVIRUSES.
PLATE 1

(Facing p. 329)

EXPLANATION OF PLATE

Fig. 1. A comparison of plaques formed by Akron +^{cr} virus (PP1) and by Akron *cr*ⁱ virus (PP5) on cystine-supplied monkey kidney cells and on monkey kidney cells not supplied with cystine. The plaques developed on medium D-grown cells for 4.0 days at 35.9°. Plates labelled 1 and 1C each received 0.30 ml. Akron PP1 at 10⁻⁵ virus concentration and plates 5 and 5C each received 0.30 ml. Akron PP5 at 10⁻⁴ virus concentration. The maintenance medium for plates 1C and 5C was AEm with L-cystine at 1.0 mM for 1 and 5 was AEm without cystine. No plaques were found on plate 1. The arrow on plate 5C points to a turbid plaque with a diameter of 4.0 mm. Seven turbid plaques were found on plate 5C.

Fig. 2. A comparison of plaques formed by Mabie +^{cr} virus (PP1a) and by Mabie *cr*ⁱ virus (PP5) on cystine-supplied monkey kidney cells and on monkey kidney cells not supplied with cystine. The plaques developed on medium D-grown cells for 4.0 days at 35.9°. Plates labelled 1a and 1aC each received 0.30 ml. Mabie PP1a at 10^{-4.4} virus concentration and plates 5 and 5C each received 0.30 ml. Mabie PP5 at 10^{-4.7} concentration. The maintenance medium for plates 1aC and 5C was AEm with L-cystine at 1.0 mM and for 1a and 5 was AEm without cystine. Seven small plaques were found on plate 1a. The rather large ill-defined lighter areas on plate 1a are not plaques; they are typical of cystine-deficient monolayers and may be found on control plates uninoculated with virus. Two turbid plaques were found on 5C; one of these with a diameter of 2.5 mm. is indicated by an arrow.

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