THE D → C CHANGE IN POLIOVIRUS PARTICLES*

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POLIOVIRUS suspensions, crude or purified, of each of the 3 serological types contain particles possessing one or other of two different, type-specific, antigenic reactivities—D or C. The differentiation and measurement of the D and C antigens have been discussed in a previous paper (Le Bouvier, 1959) in which were defined the terms " D antigen " and " C antigen " as used in this study. Their meaning is qualified by the method employed, which has been that of antigen-antibody precipitation in agar. The conclusions reached apply to the specific configurations taking part in that particular reaction. These configurations are not necessarily identical with those detected by other methods, such as complement fixation or flocculation in liquid mixtures. Nevertheless, from tests done on comparable sucrose-density-gradient fractions of purified poliovirus (Schwerdt and Schaffer, 1956 ; Schaffer and Schwerdt, 1959) it appears that the D-antigenic configuration reacting by complement fixation and that reacting by precipitation are both present on the same kind of particle, whatever may be the degree of overlap between the precise chemical groups involved (Mayer, 1957 ; Mayer *et al.*, 1957 ; Le Bouvier, Schwerdt and Schaffer, 1957). The same holds good for the C-antigenic configuration.

The evidence from absorption tests indicates that, at least in the conditions so far examined, the D and C antigenic characters are associated with different particles (Hummeler and Hamparian, 1958 ; Le Bouvier, 1959). If there is a state in which the two kinds of configuration can co-exist, with full activity, on the selfsame particle, then it would appear to be an ephemeral one ; or else, if stable, one that is present in only a small proportion of the population. Results obtained by flocculation of liquid mixtures, however, suggest that the qualification " with full activity " may be important (Smith, unpublished observation).

Besides D antigen, unheated poliovirus concentrates may also contain " spontaneous " C antigen in detectable amounts. The proportions of D and C vary from one concentrate to another ; but in most fresh preparations the quantity of C antigen is relatively small and may even be undetectable in agar-diffusion tests. When such concentrates are heated, or treated in various other ways, they are changed (Roizman, Mayer and Rapp, 1958 ; Hummeler and Hamparian, 1958 ; Roizman, Mayer and Roane, 1959). Having previously been largely or entirely D, the specific reactivity now becomes indistinguishable, by all immunological tests so far applied, from that of the " spontaneous " C antigen present in unheated crude virus preparations or in the purified sucrose-density-gradient fraction SDG^C. This alteration of character is accordingly referred to as the

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D → C change. The present paper is concerned with the following aspects of this D → C change as studied by antigen-antibody precipitation in agar: the origin of the C-antigenic particles in heated suspensions, and their relationship to the D-antigenic particles initially present; the ability of certain agents, other than heat, to produce the change or modify its course; and, finally, the kinetics and possible mechanism of the reaction.

MATERIALS AND METHODS

Agar-diffusion tests.—The reactants and general procedures have been described in the earlier paper (Le Bouvier, 1959). The reactants most commonly used were 50-fold centrifuge concentrates of Type 1 (Brunhilde) virus grown in HuLi cells (Westwood, Macpherson and Titmuss, 1957), and either the Yale Brunhilde monkey antiserum or the " standard " Brunhilde rabbit antiserum. Types 2 and 3 polioviruses were represented by the Y-SK and Leon strains, respectively.

I am indebted for monkey antisera to Dr. E. M. Opton of the Section of Epidemiology and Preventive Medicine, Yale University School of Medicine; and to Drs. F. O. MacCallum and G. P. B. Boissard of the Virus Reference Laboratory, Colindale. Some of the rabbit antisera were kindly provided by my colleagues Drs. G. M. Churcher and G. Belyavin.

The tests were of the two-dimensional kind. Both antigen and antibody reactants were allowed to diffuse into the agar. In the usual pattern of experiment, the relative D or C antigen content of various preparations was determined with a single dilution (1/8) of the " standard " serum, the serum cup being surrounded by up to eight virus cups. Besides the " standard " serum, an " anti-C " serum was used to measure changes in the amount of C antigen. This serum, obtained from a rabbit immunized with Brunhilde virus heated at 60° for 20 min., gave no detectable precipitation with D antigen at a dilution of 1/8. Agar-diffusion tests were photographed after incubation for 3 days at 37°. From the position of the leading edges of precipitates, measured on the photographs, the relative antigen concentrations were calculated on the basis of the previous findings (Le Bouvier, 1959).

Infective-virus assay.—Virus dilutions were made in Earle's balanced salt solution containing 0·25 per cent lactalbumin hydrolysate and 5 per cent tryptic digest meat broth, adjusted to pH 7·0 with 2·78 per cent sodium carbonate. Plaque counts were done in 250 ml. Pyrex rubber-stoppered bottles. The HuLi cell line, derived from cultures of human embryonic liver (Westwood *et al.*, 1957), was grown as previously described (Le Bouvier, 1959). The agar-overlay procedure was adapted from that of Hsiung and Melnick (1957). Unwashed Difco-" Bacto " agar (2·4 per cent in distilled water) was mixed, in a 45° water-bath, with an equal volume of medium containing all the ingredients at double strength, so as to give the following final concentrations in the overlay: 0·25 per cent lactalbumin hydrolysate, 5 per cent tryptic digest meat broth, and 10 per cent inhibitor-free horse serum, in Earle's saline containing 0·11 per cent sodium carbonate. The mixture was not " gassed " with CO_2. Phenol red was omitted and neutral red added (1/60,000 in the final overlay mixture). Penicillin, 100 units/ml., and streptomycin, 100 μg/ml., were included.

Cultures were used as soon as they were confluent, *i.e.*, when the glass surface of area 23 cm^2 was covered by ~5 million cells. After removal of all the liquid medium, the virus inoculum of 0·5 ml. was distributed over the cell sheet and left in contact with it for 1 hr. at 37° before introduction of 10 ml. of the agar overlay. After 1 hr. at room temperature to permit firm gelling of the agar, the bottles were incubated at 37° with the cell sheet uppermost. Plaques were usually visible on the 2nd day and were well developed by the third. Counts were made during the period between the 3rd and 5th day.

Thermal inactivation of D antigen.—Heating was carried out in an adjustable water-bath with a temperature variation of not more than ± 0·2°. In the usual type of experiment, 0·05 ml. aliquots of virus concentrate were placed in 0·75-mm. thick, acid-cleaned, soda-glass tubes 7·5 cm. in length and of internal diameter 0·75 cm. The tubes, stoppered with rubber bungs, and with a volume of contained air of ~3 $cm.^3$, were immersed for about 4/5 of their length in the water, and their contents agitated during the first 15 sec. of heating. Immersion for up to 20 min. produced no obvious condensation in the upper part of the tube. At the end of the period of heating, the tube was cooled in ice-water. Changes of volume and pressure during heating were not measured. Parallel tests using both stoppered and loosely capped

tubes showed no apparent difference in the inactivation rate of Brunhilde virus D antigen at 50°. However, when a larger volume of virus concentrate (0·5 ml.) was heated at 50° in a stoppered tube of this sort, and 0·05 ml. aliquots were removed at intervals, the inactivation rate was slowed to about one-half, despite thorough agitation of the contents.

Ultraviolet irradiation.—Brunhilde virus concentrate was irradiated in 1·5–2·5 ml. amounts in Petri dishes of diameter 9 cm. placed at a distance of 13·7 cm. from the source of ultraviolet light (UVL) and rocked at 50 cpm. Irradiation was done at room temperature (*c.* 20°). I am indebted to Dr. Elinor McCloy of the Department of Bacteriology, London School of Hygiene and Tropical Medicine, for the opportunity of using the UV lamp and for the information concerning dosage. The source of UVL was a 15-watt germicidal lamp emitting 95 per cent of its radiation in the wavelength 2537 Å. It had been calibrated by determining the rate of inactivation of coliphage T2 (Luria and Latarjet, 1947).

Other agents.—Poliovirus concentrates were tested in agar of varying pH, and subjected to freezing and thawing, drying, and treatment with mercuric chloride, merthiolate and phenol. The details are given in the section on Results.

RESULTS

Antigenic Conversion of Poliovirus Suspensions from D to C

Decrease of D antigen and increase of C antigen on heating

When poliovirus concentrates are heated at temperatures between 50° and 60°, their content of D antigen falls, while their C antigen concomitantly increases. Aliquots of a Brunhilde virus preparation were heated at 50° for varying lengths

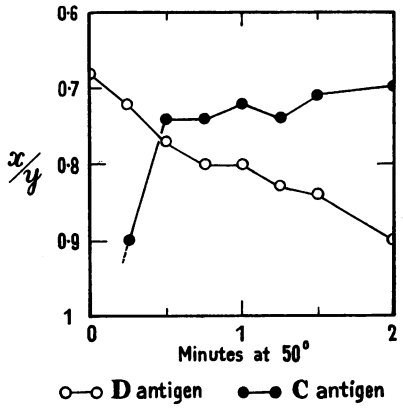

FIG. 1.—Decrease of Type 1 poliovirus D antigen and increase of C on heating at 50°
Ordinate { x = distance of leading edge of each precipitate from serum cup.
y = distance between serum and virus cups.

of time and then tested with single dilutions of 2 rabbit antisera, one containing mostly anti-D precipitins, the other anti-C. The results are shown in Fig. 1.

As the concentration of D antigen decreases, its precipitate is formed closer to the virus cup and the value for x_D/y increases. During the same period there is a rise in the concentration of C antigen. The reason for the flattening of the curve for the D antigen is not known. It is more usual, as will be seen later, for the points to fall on a straight line. The virus preparation used in this experiment consisted of a pool of three different Brunhilde virus concentrates, selected because they contained no detectable C antigen in the unheated state. The effect

seen may have been due to the presence of a relatively heat-resistant fraction in each of the three concentrates, or, more probably, to the greater resistance of one of them. Whatever the reason, the C antigen apparently increases *pari passu* with the decline of the D.

The relation between D-antigenic and C-antigenic particles

The concurrent increase in C and diminution in D antigen on heating could be coincidental, the D and C particles being independent of one another, but happening to be inactivated and activated, respectively, by equivalent degrees of heat. Alternatively, there might be a connexion between them. This could be an indirect one, the 2 kinds of particles being of separate origin. For example, " latent " precursors of C particles might depend for their activation upon the presence of some " unmasking " or inhibitor-destroying factor produced during heat inactivation of the D, or there might be a more direct connexion, C particles arising from the D particles themselves as the result of an antigenic change in their reactive surface.

That the formation of C antigen by heat is dependent on the presence of D is indicated by several consistent findings. C antigen fails to appear, or to increase in amount, in preparations containing no detectable D antigen before heating, whether the D-antigenic particles be separated by physical means, *e.g.* sucrose-density-gradient fractionation, or by specific aggregation with anti-D antibodies and removal of the precipitate by spinning. Moreover, the amount of C antigen formed on heating varies directly with the amount of D antigen initially present.

Experiments were made to try to distinguish between the possible origin of C particles from " masked " precursors in a manner only indirectly dependent on the D, and the alternative possibility that C-antigenic particles are formed directly from the D particles themselves.

Attempts to demonstrate an inhibitor of C-antigen precipitation gave negative results. Heated Type 1 virus concentrate was mixed in varying proportions with different lots of unheated homotypic or heterotypic concentrates. The amount of C precipitate formed by the heated virus was not reduced, even by those preparations which contained no " spontaneous " C antigen and which might have been expected to contain an excess of free inhibitor, if any such existed.

The possible presence of " masked " C-particle precursors was investigated by incubating Brunhilde virus concentrate, lacking detectable " spontaneous " C antigen, with an " anti-D " serum made by absorbing a Brunhilde monkey antiserum with the same virus heated at 60° for 20 min. The resulting D antigen-antibody aggregate was removed by spinning in the No. 40 Spinco rotor at 10,000 r.p.m. for 30 min. (mean RCF = 6,600 *g*). A control virus-diluent mixture was similarly treated. It was found that the capacity to form C antigen on heating was sedimented along with the D antigen-antibody aggregate from the virus-serum mixture ; whereas in the control mixture it remained in the supernatant. The C-particle precursor had either been specifically precipitated by the anti-D antibody, or been carried down mechanically with the D aggregate.

A similar experiment was done to test the effect of C antigen-antibody aggregation, as opposed to D. An " anti-C " serum was made by absorbing the Brunhilde monkey antiserum with unheated Brunhilde virus concentrate. This " anti-C " serum was mixed with heated Brunhilde virus in the presence of homologous

unheated concentrate containing no detectable " spontaneous " C antigen. Control suspensions consisted of the 2 virus preparations, either alone or combined, mixed with diluent instead of serum. The mixtures were spun at 10,000 r.p.m., and the supernatant fluids and deposits tested before and after heating. An increase of C antigen on heating was observed only in those mixtures in which unheated virus had been included : in each case it was seen only in the supernatant, and the amounts formed were comparable in test and control mixtures. There was therefore no reason to suspect sedimentation of a " masked " C-particle precursor as the result of mechanical entanglement or non-specific adsorption by the C antigen-antibody aggregate.

A direct conversion of D-antigenic particles into C-antigenic particles appears to be the simplest explanation of these findings.

Production of the D → C Change by Different Agents

Influence of pH on D and C precipitation

The solution used for preparing virus concentrates and for diluting all reactants was 0·14M sodium chloride buffered at pH 7·3 with 0·01 M phosphate. When exposed to the air, the pH of this weakly buffered solution and of the virus concentrates fell to about 7·1. On heating, the pH rose again to around 7·3 or higher (up to 7·6). To determine the effect of such variations on the precipitation reactions of poliovirus D and C antigens, tests were done in agar containing citrate-phosphate-borate buffer solutions of different pH (Theorell and Stenhagen, 1939). The pH of these solutions was determined by glass electrode. B.D.H. " Universal " indicator was added to the agar to detect any gross changes that might occur during the 3-days' incubation at 37°. The D and C antigen preparations consisted of 50-fold concentrates of the representative strain of each virus type, unheated or heated at 60° for 20 min., respectively. These were tested against selected single dilutions of monkey and rabbit antisera. The results are given in Table I.

TABLE I.—*Effect of pH on Poliovirus D and C Precipitation Reactions*

pH of agar	Poliovirus antigen preparations					
	Type 1		Type 2		Type 3	
	D	C	D	C	D	C
⩽3	−	−	−	−	−	−
4–5	+	±	±	−	+	−
6	+	+	+	−	+	−
7	+	+	+	+	+	+
8	+	+	+	+	± (→)	+
9–10	± (→)	+	tr. (→)	+	tr. (→)	+

D = the D antigen in unheated 50-fold virus concentrates.
C = the C antigen in the same concentrates heated at 60° for 20 min.
\+ = precipitate maximal, or nearly so, as judged by its density and distance from virus cup (see text).
± = precipitate about ½ to 1/4 of maximal amount.
tr. = trace of precipitate (< 1/4 of maximal amount).
− = no detectable precipitate.
(→) = increase in amount of C precipitate given by the unheated concentrate. (This is the only symbol which refers to the C-antigen content of *unheated* virus concentrates.)

Variation in the amount of precipitate was apparently related to the effect of pH changes on antigen rather than on antibody : the fainter the precipitate, the smaller was its distance from the virus cup.

Near the pH range of the heating experiments (pH 7·3–7·6), there appeared to be no significant alteration in the precipitation reactions of the representative strains of the 3 poliovirus types, except for the incipient reduction of the Type-3 D precipitation at around pH 8. With greater deviations from neutrality, however, a consistent pattern of impairment was observed with all the strains, the D activity being more sensitive to an alkaline, the C to an acid pH. This deleterious effect of low pH on the C precipitation was especially marked with the strains of Type 2 (Y-SK) and Type 3 (Leon). Strains of the same type may not behave exactly alike : in a few experiments with the MEF-1 strain of Type 2 virus, the D-antigen precipitation appeared to be as susceptible as the C to a pH in the region of 6. Below pH 3, the reactants in all cups appeared coagulated. In the pH range 9–10, the decrease in the amount of D precipitate given by the unheated concentrates of each type was accompanied by a rise in the C reactivity, suggesting a conversion of D antigen to C. The amount of precipitate formed by the heated concentrates remained unaffected by this alkaline pH. At higher pH levels (> 12) all poliovirus precipitating activity was abolished, C as well as D.

Concentrations of OH^- ions insufficient to effect the D $\rightarrow$ C change during 3 days at 37° will accelerate the reaction in Brunhilde virus at higher temperatures. Virus concentrate was mixed with equal volumes of veronal-buffer solutions of different pH (7·0–9·0) and heated at 50°. None of the mixtures showed any increase in C antigen before heating. But there was a progressive rise in the D $\rightarrow$ C conversion rate at 50° over this pH range : the D-antigen content was reduced to 1/10 in 3 min. at pH 7·0, and in 30 sec. at pH 9·0 ; the time for the control mixture with phosphate-saline diluent (pH 7·3) was $2\frac{1}{2}$ min.

D $\rightarrow$ C conversion of poliovirus by heat

Reproducibility of heating experiments.—The kinetics of the heat-inactivation reaction of Type-1 poliovirus D antigen are considered in more detail in a later section. As was mentioned earlier, in some cases the reaction rate may not be uniformly exponential (Fig. 1) ; but with most freshly made preparations the inactivation curve takes the form of a single straight line, as seen in Fig. 2. This shows the course of D-antigen inactivation at 50° in a suspension of Brunhilde virus. The virus was heated both as the undiluted 50-fold concentrate and as a 1 in 5 dilution in phosphate-buffered saline. Four replicate tests were made with each preparation. The points in Fig. 2 represent the individual readings. Straight lines were fitted by the method of least squares. An analysis of variance showed that the accord with linearity, for the 50-fold concentrate, was good within the limits of experimental error ($P > 0·2$). There was no significant difference between the slopes of the two lines ($P > 0·2$) ; but their intercepts on the ordinate were significantly different ($0·01 > P > 0·001$).

Variability of D $\rightarrow$ C conversion rate and its acceleration by horse serum.—With the majority of Brunhilde virus concentrates made by the method described, the D-antigen content was reduced to 1/10 after 1 to $1\frac{1}{2}$ min. at 50°. With occasional preparations, however, the time required might be as little as 1/4 min. or as much as 4 min. or even longer. Comparative tests of D-antigen inactivation rates were

always made on fractions derived from a given virus concentrate : these were heated simultaneously, where possible, or else within the space of a few hours. The inactivation rate is evidently affected by many factors which, even if known, may be difficult to standardize, particularly in crude concentrates of the kind used in this study. One example may be mentioned here : it illustrates the possible effect on the D → C conversion rate of the horse serum used in the culture medium.

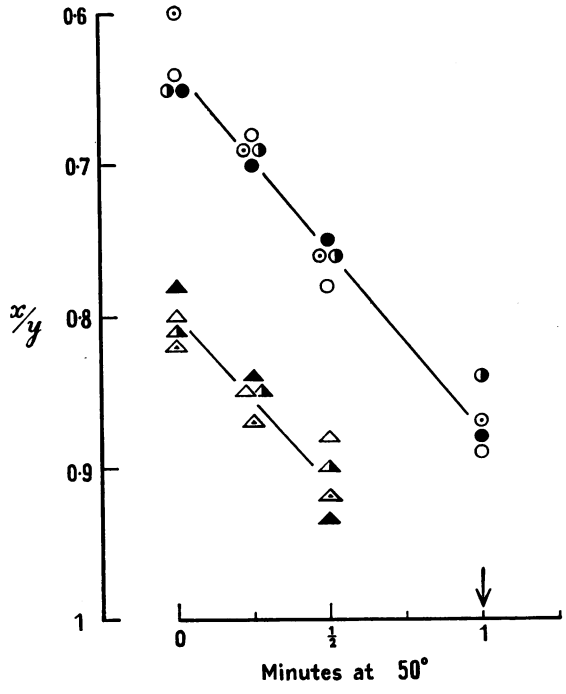

FIG. 2.—Replicate heat-inactivation tests of Type-1 D antigen

Ordinate { x = distance of leading edge of precipitate from serum cup.
y = distance between serum and virus cups.

Circles—50-fold virus concentrate
Triangles—1 in 5 dilution thereof } 4 replicate tests of each

Arrow indicates that leading edge of precipitate has entered virus cup

A batch of Brunhilde virus concentrate was divided into 2 lots, one of which was " washed " by two cycles of dilution in 10 vols. of phosphate-saline and sedimentation at 39,000 r.p.m. for 2 hr., the final pellet being resuspended in diluent to the original volume. The " unwashed " and " washed " concentrates were each divided into two portions, to one of which 1/10 volume of diluent was added, to the other 1/10 volume of unheated horse serum, as used in the culture medium. The 4 fractions were left for 1 hr. at room temperature (18°) and heated in parallel at 50°. The D-antigen inactivation slopes are shown in Fig. 3 : " washing " resulted in a slower loss of D antigen at 50° ; after addition of horse serum, the inactivation rates for both " washed " and " unwashed " virus were increased and became about equal. Heating the serum at 60° for 30 min. did not appear

to alter this effect. The degree of acceleration of the D → C change varied with the batch of horse serum.

Abolition of C reactivity at temperatures above 70°.—The antigenic change from D to C was produced by short exposure to temperatures at or just above 50°. D-antigen preparations of Types 2 and 3 tended, on heating, to be more rapidly changed than Type 1 preparations. But there were also marked divergences between strains of the same type, and indeed between different preparations of the same strain. In the range between 50° and 60°, poliovirus C antigen of Types 1 and 2 appeared relatively stable, that of Brunhilde virus showing little or no reduction even after heating at 60° for 2 hr. With Type 3 virus, however, there was usually some decrease in C antigen after 20 min. at 60°. At higher temperatures the C antigen of all 3 types, and with it all poliovirus precipitating activity, was lost. Differences between the representative strains of the 3 types were

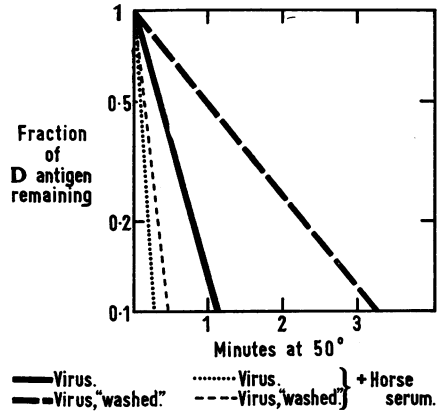

FIG. 3.—Acceleration of D-antigen inactivation in a Brunhilde virus concentrate by horse serum.

readily demonstrable, even with the rough test method employed. Aliquots of 50-fold virus concentrates were heated for 1-min. periods at various temperatures from 60°–100°. The temperatures which abolished all detectable C activity in this time were ~ 75° for Type 3, ~ 80° for Type 2, and ~ 85° for Type 1.

Infectivity loss and D → C change induced by heat and ultraviolet light

Heat and ultraviolet irradiation can both bring about the antigenic change from D to C. But whereas with heat the inactivation of virus infectivity and the decline of D antigen proceed at similar rates, with UV irradiation the abolition of infectivity is much the more rapid (Le Bouvier, 1955 ; Roizman *et al.*, 1959). Further evidence of this contrast is presented in Figs. 4 and 5. In one experiment, the loss of infectivity on heating occurred more rapidly, in another more slowly, than the change in the D antigen, with about a 2-fold difference in the reaction rates ; with UV irradiation, however, infectivity dropped to 10 per cent of its original level about 30 × more quickly than D reactivity. The findings are possibly related to differences in the mechanism of virus degradation and may suggest a link between the D → C change and the way in which the virus-particle infectivity is destroyed by heat as distinct from UV light. In Fig. 5 there is some suggestion

of a " multi-hit " type of inactivation curve for the D antigen. Its significance is not clear. With a different concentrate, irradiation of the D antigen produced a linear inactivation, and this was also the finding of Roizman, Mayer and Roane (1959).

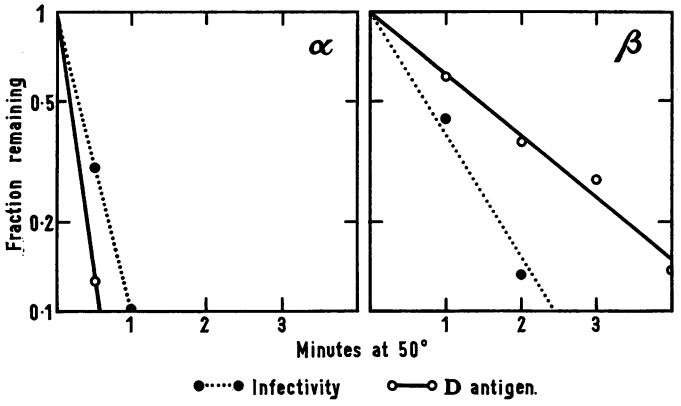

FIG. 4.—Infectivity loss and D-antigen inactivation induced in Brunhilde virus by heat

With the virus batch *a*, infectivity titrations were done before concentration (titre before heating = $10^{8\cdot1}$ plaque-forming units per ml.)

With *β*, infectivity titrations were done on the 50-fold concentrate (titre before heating = $10^{9\cdot9}$ plaque-forming units per ml.).

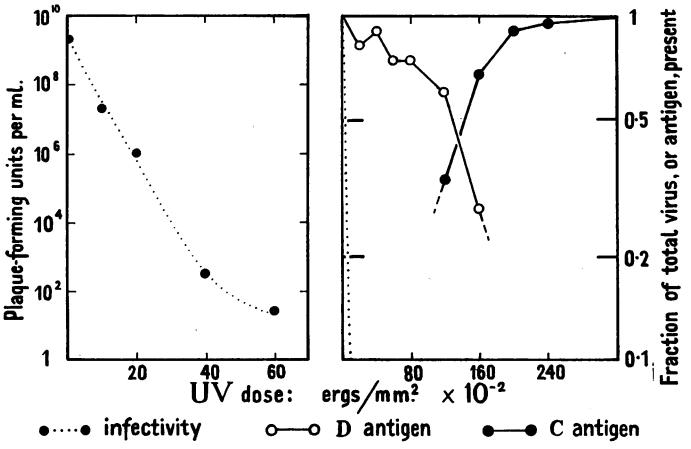

FIG. 5.—Infectivity loss and D → C change induced in Brunhilde virus by ultraviolet light. Infectivity titre of 25-fold virus concentrate before irradiation = $10^{9\cdot3}$ plaque-forming units per ml.

No C antigen detectable with UV dose $\leqslant$ 8,000 ergs/mm.².
No D antigen detectable with UV dose $\geqslant$ 20,000 ergs/mm.².

Treatment of 2 ml. of a 25-fold Brunhilde virus concentrate with a dose of UV irradiation of 600 ergs/mm.² resulted in a 90 per cent decrease in infectivity, whereas $\sim$ 20,000 ergs/mm.² were needed to effect a similar percentage reduction of the D antigen. As with heat, a more prolonged irradiation with UVL led to the loss of all C reactivity : in this preparation no C antigen was detectable after

a dose of 140,000 ergs/mm.^{2} To determine whether some third kind of specific poliovirus precipitinogen had been formed, which was unable to react with antibodies present in the " standard " or " anti-C " Type-1 rabbit sera, a rabbit was immunized with the same Brunhilde virus concentrate irradiated with a dose of 160,000 ergs/mm.^{2} Two intravenous injections of 1 ml. were given 3 weeks apart. Undiluted serum taken before, and 1 week after, the second injection contained small amounts of anti-C antibody, but gave no precipitate with either D antigen or the UV-irradiated concentrate used for immunization. The low levels of C precipitin were probably evoked by traces of C antigen remaining in the inoculum, even though it did not contain enough to form a detectable precipitate in agar.

Induction of the D → C change by agents other than heat, UVL and hydroxyl ions

This section deals briefly with some of the ways in which different physical and chemical agents may influence the poliovirus D → C conversion. In testing chemical compounds two methods were employed, of which examples are given below : incorporation of the substance in the agar ; and pre-incubation of virus (or serum) with the agent, usually for 1 hr. at 37°, before introduction of the mixture into the cups. In a few experiments the virus was spun down after incubation, and the pellet resuspended in diluent. Test plates were held at 37° or at 4°.

The process of D → C antigenic conversion was brought about, or modified, by a variety of agents and procedures. With some, the effect appeared to be independent of temperature : *e.g.*, lyophilization resembled drying at higher temperatures in causing the D → C change in concentrates of all 3 poliovirus types ; the dried virus in each case was resuspended in water at 4°.

Other agents apparently interacted with temperature in bringing about the D → C change. In sufficient concentration they accelerated the reaction, so that it could be observed at temperatures at which it was usually too slow to be practically measurable (4° to 37°). In somewhat lower concentrations they increased the rate of D → C change in virus heated at 50°. In contrast to these " accelerators ", some agents were found to retard the process, while others again appeared to be " neutral ".

Examples of D → C accelerating agents were mercuric chloride, merthiolate (sodium ethylmercurithiosalicylate) and phenol. These are considered below in more detail. The concentration of Brunhilde virus D antigen was reduced by exposure to acetone or ethanol for 5 hr. at 4°, or to 0·1 per cent potassium permanganate for 1½ hr. at 37° ; but these compounds were not sufficiently studied to establish a concomitant rise in C antigen. The action of horse serum has been mentioned in an earlier section.

The rate of D → C change in a Brunhilde virus concentrate at 52° was slowed if the virus was suspended in hypertonic solutions of sodium chloride. The degree of retardation increased progressively with the molarity : in 0·5 M solution the time taken for the D antigen to fall to 1/10 of its original concentration was about 10 × as long as in 0·14 M solution. At salt concentrations higher than 0·5 M, the rate of D → C change at this temperature was too slow to be conveniently measurable. Suggestive evidence was also obtained of retardation of the change in Brunhilde virus at 50° after treatment with formaldehyde at pH 7 (0·1 per cent for 5 weeks at 4°), or after incubation with 0·01 per cent L-cystine for 30 hr. at 37°, a procedure which has been found to stabilize the infectivity of a heat-sensitive line of Brunhilde virus (Pohjanpelto, 1958).

Exposure of Brunhilde virus to ether or chloroform for 5 hr. at 4° caused no detectable reduction in D antigen. The D → C conversion rates at 50° of virus so treated were not determined. The D-antigenic character of strains of each virus type was apparently not affected by freezing and thawing of virus concentrates up to 5 times, but this treatment caused aggregation of other, presumably non-viral, components, which could then be separated by spinning, leaving a clearer virus reactant. Pre-incubation of Brunhilde virus at 37° for 3 days (followed by the usual 3-day test at 37°) produced no detectable diminution in D antigen, nor did it lead to a more rapid D → C conversion at 50°. That some such effect might be expected, however, if the sojourn at 37° were further prolonged is suggested by the fact that 4 months' storage at 4°, though leaving the D-antigen concentration still unaltered, did result in an increased rate of change from D to C on heating, as compared with the same Brunhilde virus concentrate stored at −23°.

The results of incorporation tests with mercuric chloride and merthiolate, and of a pre-incubation test with phenol, are presented in Tables II and III. These and other agents causing detectable changes in the precipitation reaction evidently act largely, if not entirely, on the antigen; for diminution in the amount of precipitate is accompanied by a shift of the band towards the virus cup. Selected dilutions of monkey and rabbit antisera were used.

TABLE II.—*Effect of Mercuric Chloride and Merthiolate on Poliovirus D and C Precipitation Reactions*

Concentration of agent in agar (% w/v)	Poliovirus antigen preparations: Type 1 (Brunhilde) D	Type 1 (Brunhilde) C	Type 2 (MEF-1) D	Type 2 (MEF-1) C	Type 3 (Leon) D	Type 3 (Leon) C
Mercuric chloride:						
None	+	+	+	+	+	+
0·002	− (→)	+	− (→)	+	− (?→)	±
0·01	− (→)	+	−	tr.	−	−
Merthiolate:						
None	+	+	+	+	+	+
0·001	+	+	+	+	tr. (?→)	±
0·01	tr. (→)	+	− (→)	+	−	−
0·1	− (→)	+	− (→)	±	−	−

Test read after 3 days' incubation at 37°.
D = the D antigen in unheated 50-fold virus concentrates.
C = the C antigen in the same concentrates heated at 60° for 20 min.
\+ = precipitate maximal, or nearly so, as judged by its density and distance from virus cup (see text).
± = precipitate about ½ to 1/4 of maximal amount.
tr. = trace of precipitate (< 1/4 of maximal amount).
− = no detectable precipitate.
(→) = increase in amount of C precipitate given by the unheated concentrate. (This is the only symbol which refers to the C-antigen content of *unheated* virus concentrates.)

In the experiments with mercury compounds summarized in Table II the chelating agent versene (disodium dihydrogen ethylene-diamine-tetra-acetate) was left out of the agar. In another experiment it was incorporated in 0·07 per cent final concentration in the plate containing 0·01 per cent merthiolate. As a

result, the Type 1 and Type 2 strains gave maximal D precipitation (+) comparable with that seen in the control plates. With the Type 3 strain, however, the most sensitive to the action of merthiolate, this concentration of versene was not fully protective and the D precipitation was submaximal (±). The activity of versene in countering the D → C accelerant effect of merthiolate is presumably related to its function in safeguarding the immunizing potency of merthiolate-preserved poliovirus vaccine (Davisson *et al.*, 1956). Versene was also effective against the D-modifying action of mercuric chloride.

A plate containing 0·1 per cent merthiolate, but kept at 4° instead of 37°, showed no differences from the control beyond a slight reduction in the amount of D precipitate formed by the strains of Types 2 and 3.

TABLE III.—*Effect of Phenol on the Precipitation Reactions of Brunhilde Virus Antigens*

Concentration of phenol in virus-phenol mixture (% w/v)	Antigen preparation	
	D	C
None	+	+
0·5	+	+
1·0	±	+
1·5	tr. (→)	+
2·0	− (→)	+
2·5	−	tr.

Virus-phenol mixtures incubated at 37° for 1 hour before being placed in the cups.
Test read after 3 days at 37°.
Symbols mean the same as in Table II.

Table III shows the result of a test of Brunhilde virus 50-fold concentrate, unheated or heated at 60° for 20 min., of which aliquots were pre-incubated for 1 hr. at 37° with equal volumes of varying dilutions of phenol in phosphate-saline. The pH of the initial 5 per cent phenol dilution was adjusted to 7·1 with 1 N NaOH. Phenol concentrations below 1 per cent, which did not induce the D → C change at 37°, were nevertheless effective, in proportion to their strength, in hastening the conversion of Brunhilde virus at 50°. Treatment with water-saturated phenol at 4°, as in the extraction of virus ribonucleic acid, degraded both D and C antigens beyond recognition.

Kinetics of the D → C Change

The modification of poliovirus Type-1 D antigen by heat has some of the attributes of a unimolecular, or of a pseudo-unimolecular, reaction. The inactivation rate was found to be exponential, at least over the greater part of the measurable course of the reaction, and the slope of the inactivation curve was independent of the initial D-antigen concentration within the narrow range that could be tested (Fig. 2). This linear inactivation can also be seen in Fig. 6, which illustrates the marked temperature dependence of the D → C conversion.

Brunhilde virus 50-fold concentrate was heated at different temperatures between 48° and 52°, and the various reaction rate constants calculated from the equation $k' = 1/t$, where t is the time in minutes at which the fraction of D antigen left is 0·37, or e^{-1}. There was about a 2·7-fold increase in the value of k' for each

1-degree rise in temperature. A corresponding gradation, not shown in Fig. 6, was observed in the rate of emergence of C antigen at these different temperatures. Over the 4-degree span within which experiments were feasible, a linear relationship existed between the logarithm of the rate constants for D-antigen inactivation and the reciprocal of the absolute temperature (Fig. 7).

Although the evidence so far obtained suggests that the D → C change may be a first-order reaction with respect to concentration as well as time it must be admitted that the range both of temperature and of concentration in which it could be studied was exceedingly small. The preparations used in these experi- experiments were unpurified and no electron-microscopic particle counts were attempted. A rough estimate of the mass of virus present may be made, however, by assuming that there were between 50–500 non-infective particles for each one capable of producing a plaque, on the basis of the findings with polio-

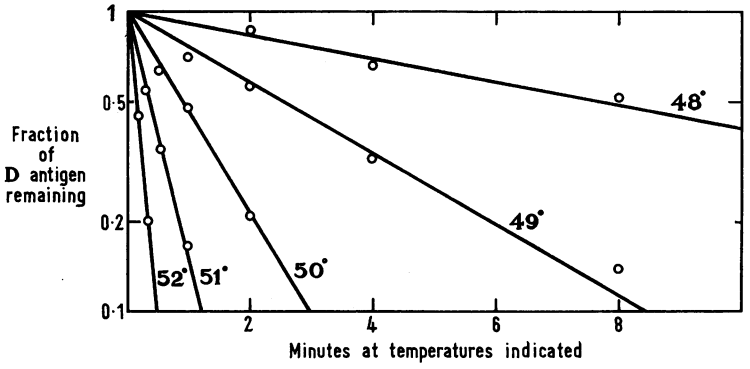

FIG. 6.—Inactivation of poliovirus Type-1 D antigen at different temperatures.

virus grown in monkey-kidney and human-amnion cells (Schwerdt and Schaffer 1955 ; Schwerdt and Fogh, 1957). The infectivity titre of Brunhilde virus liquids before concentration was usually about 10^8 plaque-forming units/ml. Taking the virus-particle mass as $1{\cdot}1 \times 10^{-17}$ gm. (Schaffer and Schwerdt, 1959), an average 50-fold Brunhilde virus concentrate would therefore be expected to contain $10^{11.4}$—$10^{12.4}$ particles/ml., or a virus mass of between 3 and 30 μg/ml.

There have been a number of reports of apparently first-order thermal inactivation reactions with different viruses (*e.g.*, Price, 1940 ; Lauffer, Price and Petre, 1949 ; Kraft and Pollard, 1954 ; Youngner, 1957 ; Kaplan, 1958 ; Luria, 1953 ; Pollard, 1953). For purposes of comparison, therefore, thermodynamic constants were calculated for the D → C change in poliovirus : $\Delta H^{\ddagger}$ (taken as $\simeq E_{exp.}$, the experimental activation energy, derived from the data in Fig. 7) was found to be 203 kcal./mole, and $\Delta S^{\ddagger}$ to be + 570 entropy units. Whatever the exact significance of these values may be, the activation of D particles during the antigenic conversion of poliovirus suspensions from D to C appears to be associated with a large total heat energy change and a correspondingly large increase in entropy.

DISCUSSION

By physical means, poliovirus suspensions of a given strain can often be divided into fractions which react predominantly either as D or as C antigens in direct

tests of complement fixation or precipitation in agar (Mayer *et al.*, 1957 ; Le Bouvier *et al.*, 1957 ; Roizman *et al.*, 1958, 1959). It has also been possible to separate the two kinds of antigenic particles by specific absorption, at least from certain sorts of virus suspension and using selected animal antisera (Hummeler and Hamparian, 1958 ; Le Bouvier, 1959). Most of the particles in such absorbed preparations possess, in effect, either D reactivity, or C, but not both at the same time.

When suspensions containing D antigen are heated, C antigen appears, or, if already present, increases in amount. The present experiments indicate that the precursors of C-antigenic particles are either the D particles themselves, or particles that have so far proved to be indistinguishable from them. In the absence of contrary evidence, there seems little reason to consider these precursors

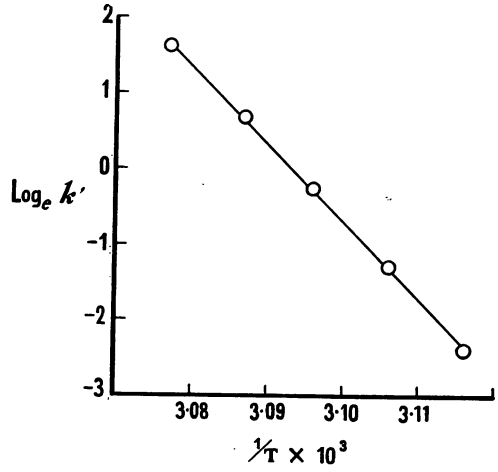

FIG. 7.—Arrhenius plot constructed from data shown in Fig. 6.
k' = inactivation rate constant of D antigen at absolute temperature T.

as " masked " C particles, or as complexes of C particles with some inhibitor. All the results so far obtained are consistent with the hypothesis that particles with D reactivity are themselves changed by heat into C-antigenic particles.

The D → C change has the form of a unimolecular, or of a pseudo-unimolecular, reaction. In most Brunhilde virus 50-fold concentrates the change is measurable in about 90–95 per cent of the particles. In this majority of particles the D-antigen inactivation curve is usually exponential. The rate-determining step in the reaction could be the breaking of a secondary hydrogen bond, such as may occur in certain protein denaturations (Laidler, 1958), or an oxidation, as has been suggested by Pohjanpelto (1958) for the heat inactivation of poliovirus infectivity. Whatever the nature of this reaction may be, it can evidently be precipitated by a number of factors. These presumably work in different ways to produce the same result—an altered architecture of the " shell " of protein sub-units which constitutes the external layer of the D-antigenic particle (Finch and Klug, 1959). Heat and ultraviolet light might be expected to act by disrupting interhelical linkages ; phenol, by taking the place of amino groups in forming hydrogen bonds with

carbonyl groups; an increase in OH^- ion concentration, by causing ionization changes; mercuric and ethylmercuric ions, as also the foregoing agents, by severing disulphide bonds. Lyophilization, where the effect is probably due to oxidation during the drying, has been shown to have a strong splitting action: the virus particles, after resuspension in water, are found to have dissociated into fragments (Hampton, 1955; Ehrenberg and Polson, 1958).

As to the way in which the change in antigenic surface would be determined by the antecedent chemical events, various possible mechanisms can be imagined. There is at present no balance of evidence in favour of any particular one. Perhaps the simplest supposition would be that the critical reaction makes irreversible a change in the structure or arrangement of protein helices which can be initiated by several different means. Any proposed explanation of the antigenic differences between D and C particles would have to take into account the observed conjunction of deficient ribonucleic acid (RNA), altered particle structure, and C reactivity (Schwerdt, 1957; Le Bouvier *et al.*, 1957) and would depend on the answers to the following questions, among others: Can virus particles be antigenically D though lacking nucleic acid, a possibility suggested by the finding of non-infective, relatively slowly sedimenting, D-antigenic particles on density-gradient fractionation of purified virus (Roizman *et al.*, 1958)? Conversely, do particles ever acquire the C-antigenic character while retaining their RNA? Are the sub-units, and groups of sub-units, from which the poliovirus D particle is built up, themselves antigenically C? If so, do they require the presence of virus RNA to be assembled into particles of D-antigenic configuration?

SUMMARY

The antigenic character of poliovirus suspensions can be modified by heat, a process referred to as the D → C change. The nature and kinetics of this change have been studied by specific precipitation in agar, using centrifuge concentrates of tissue-culture virus and monkey or rabbit antisera.

The D → C change in the reactivity of a poliovirus suspension most probably reflects a conversion of the virus particles from the D-antigenic form to the C. This change is produced by short exposure to temperatures between 50° and 60°. Between 75° and 90°, depending on the strain of virus, the C-antigenic activity is rapidly destroyed. The D → C conversion of poliovirus can be brought about at lower temperatures (4°–37°) by a variety of agents, including ultraviolet light, drying, mercury compounds, phenol and an alkaline pH.

The heat modification of poliovirus Type-1 D antigen appears to be a unimolecular, or a pseudo-unimolecular, reaction, in which activation of the D particles is associated with a large heat energy change and a corresponding increase in entropy. It is suggested that various factors may promote the D → C change by causing a distortion of the protein structure and facilitating an irreversible reaction in the D particle.

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