TABLE I. Plasma Cholesterol and Adrenal Data for Control and Experimental Rats.

No. animals	Day	Wt, g	Plasma cholesterol, mg/100 ml	Wt adrenals mg	Wt adrenals mg/100 g	Glomerulosal width, ocular micrometer units
			Control animals			
1	13	181	75	37	20	18
1	19	180	64	28	16	18
1	26	214	58	39	18	18
1	36	365	61	54	15	18
			Experimental animals			
1	13	147*	324	31	21	26
2	15	154*	452	34	22	30
3	19	175*	401	39	22	25
2	26	162†	492	41	25	22
2	36	193	161	44	23	18
2	61	301	98			20

* These animals exhibited ascites and tissue edema, so that body weights are too high and relative adrenal weights too low.

† One of these animals had a diuresis on day 25 and still exhibited considerable tissue edema.

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Survival of Polioviruses at Elevated Temperatures (60°-75°C).* (25859)

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It is generally stated that poliovirus is rapidly destroyed at temperatures slightly exceeding 60°C(1,2). Kaplan and Melnick (3) reviewed the relevant literature and added results of their own showing that even when suspended in milk or cream, which exhibit a stabilizing effect, poliovirus from human feces as well as stock strains of rodent-adapted viruses were inactivated after heating for 30 minutes at 61.7°C. Infectivity of such preparations was also eliminated by heating at 71.1°C for much shorter intervals

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(15-18 sec). Subsequently Stanley and his co-workers(4,5), Youngner(6) and Lwoff(7) reported that at lower temperatures variant forms exist in stock suspensions of poliovirus which differ markedly in respect to thermosensitivity. In this communication results will be presented that reveal the presence of variants more thermoresistant than those previously described among populations of all 3 types of poliovirus. These results were obtained in a series of experiments initiated after unexpected multiplication of poliovirus Type II had been encountered in safety testing of a suspension heated at 65°C. It had been intended to employ this suspension as antigen in skin tests on human beings.

Procedures. Cell cultures. Primary tube and bottle cultures(8) of trypsinized human amnion and monkey kidney cells§ were incubated at 35°-37°C. Tube cultures were maintained in a roller wheel. Cultures of amnion cells when inoculated were more than 14 days old(9). For cell outgrowth either bovine amniotic fluid medium (BAF)(10) or Melnick's lactalbumin hydrolysate(1) medium were used. For maintenance, only BAF was employed. *Stock suspensions of virus.* Bottle cultures of monkey kidney cell cultures were seeded with undiluted suspensions of Mahoney (Type I), MEF_1 (Type II) and Saukett (Type III) viruses, and 3 serial passages of each were carried out. Prior to inoculation media used for outgrowth were withdrawn and replaced with medium 199. In the 2nd and 3rd passages at time of this substitution the cells were washed once and 5 times respectively with Hanks' solution or medium 199. Cultures employed in preparation of viruses for Exp. 3-6 were, in addition, washed once after inoculation and adsorption of virus. Stock suspensions consisted of fluids of the 3rd passage that were pooled and centrifuged at 2,000 rpm for 30 minutes at 4°C. Aliquots of 0.5-1.0 ml were distributed in ampoules which were glass-sealed and stored in the CO_2 box. As judged by color of the medium, pH of these aliquots did not vary beyond the range of 7-7.6. *Heating.* One of 2 water baths was employed, depending upon temperature range desired. In experiments in which effect of temperatures of 65°C or lower was studied an Elconap model 420-1‖ of large water capacity was used. Higher temperatures were provided by an Electric Hotpack bath model No. 301¶ with small water capacity. Temperature of bath was checked at 3 different loci. Readings of the 3 thermometers did not vary by more than 0.5°C. One of them was calibrated and found to be accurate within 0.05°C. Bulb and adjacent 5 cm of this thermometer were placed in a vessel comparable to that containing the virus suspension. This vessel was sealed at the point where the thermometer extended beyond the lip. Heating of viral suspensions was effected as follows. A number of ampoules were rapidly thawed in the hand and placed in an ice bath along with the calibrated thermometer. These materials were then completely immersed in the water bath at desired temperature. Time and temperature of exposure as indicated by the calibrated thermometer and checked by the 2 others were recorded. Ampoules were then replaced in the ice bath until contents were added to cell cultures. *Inoculation and maintenance of test cultures.* For each determination, contents of 2 ampoules were pooled and aliquots tested for presence of virus. As routine, amnion cell cultures were each inoculated with 0.1 ml of undiluted fluid. In one experiment 0.6 to 1.5 ml was tested. Cultures were observed at intervals for at least 53 days. Nutrient fluid (BAF) was replaced whenever pH fell below 7.0. Fluids from cultures showing questionable cytopathic changes were tested for infective virus by addition to fresh amnion cultures. In one experiment (No. 2) fluids from cultures exhibiting no cellular changes were also tested with negative results. *Controls.* In each experiment uninoculated control cultures ranging in number from 3 to 12 were included. These were treated in the same manner as inoculated cultures. In none was evidence of viral multiplication observed. *Viral assay.* Serial 10-fold

§ Cell suspensions obtained from Okatie Farms, Bluffton, S. C.

‖ Electric Heat Control Apparatus, Newark, N. J.

¶ Electric Hotpack Co.

TABLE I. Thermal Inactivation Poliovirus Type II.

	Temperature													
	55°C				60°C				65°C				75°C	85°C
	Time exposed (min.)													
Exp. No.	10	20	30	60	10	20	30	60	10	20	30	60	60	60
1*	7/12‡	4/10	4/10	1/10	1/10	0/10	0/10	0/10	1/10	0/10	1/10	4/10§		
2*	15/15			12/15	1/15			1/15	3/15			1/15		
3†												1/15		
4†													1/15§	
5†														0/15
6†													0/3 ‖	

* Titer of virus prior to heating: $10^{7.3}$ TCD_{50}/ml.
† *Idem* $10^{6.5}$ " .
‡ No. tubes positive/No. tubes inoculated.
§ Identified by neutralization test.
‖ Each tube inoculated with 0.6-1.5 ml rather than with 0.1 ml of heated suspension.

dilutions of the suspension were prepared in Hanks' solution or medium 199. Each of 5 replicate tube cultures of amnion cells was inoculated with 0.1 ml of each dilution. Infectivity titers were expressed as the log TCD_{50}/ml of the undiluted suspension. Plaque morphology was studied in bottle cultures of amnion cells following procedure described by Frothingham(8). *Neutralization tests* were done in amnion cultures employing 100 TCD_{50} of virus and a final volume of diluted type specific poliovirus antiserum and virus suspension of 0.2 ml.

Results. The results of heating 2 different suspensions of Poliovirus Type II at different temperatures for varying intervals are summarized in Table I. Evidently viral infectivity was not completely destroyed within the temperature range 55-75°C when heated for 1 hour—the longest interval studied.

Comparable experiments were carried out with suspensions of Type I and III polioviruses but temperature range was limited to 65°-85°C. Again it became apparent (Table II) that very small amounts of virus may remain capable of initiating infection of cell cultures after heating at 65°C for 1 hour. No evidence of survival, however, was obtained after heating aliquots of same suspensions for 1 hour at 75° or 85°C.

That exposure to temperatures of 55°-65°C was not without effect on surviving viral units was indicated by prolongation of the interval between inoculation and first appearance of cytopathic changes in infected cells. Occasionally CPE was first noted only after the third to the fifth week (Table III). In virus exposed to 55°C about one-third of the cultures exhibited delayed CPE. Contrasting sharply with these findings was prompt appearance of CPE in cultures inoculated with unheated virus. Thus in repeated titrations of the stock suspensions CPE always became evident at the endpoint dilutions within 6 days.

That the greatly enhanced resistance of these rare variants to heat might be genetically determined is an obvious hypothesis. Two experiments were carried out to discover whether progeny of the Type II virus that survived heating for 1 hour at 75°C were more thermoresistant than components of the

TABLE II. Recovery of Poliovirus after Exposure to Various Temperatures for One Hour.

	Temp.	65°C				75°C	85°C
	Exp. No.	1	2	3	6	4	5
Poliovirus type	1			1/15*	0/3†	0/15	0/15
	2	4/10	1/15	"	" †	1/15‡	"
	3			0/15	1/3†	0/15	"

* No. tubes positive/No. tubes inoculated.
† Each tube inoculated with 0.6-1.5 ml instead of 0.1 ml.
‡ Identified by neutralization.

Titer of virus prior to heating: Type 1: $10^{7.3}$ TCD_{50}/ml. Type 2 exp. 1 & 2: $10^{7.3}$ TCD_{50}/ml. Type 2 exp. 3-6: $10^{6.5}$ TCD_{50}/ml. Type 3: $10^{7.3}$ TCD_{50}/ml.

TABLE III. Time-lapse between Inoculation of Heated Poliovirus Type II and First Appearance of Cytopathic Changes.

Exp.	Temp. (°C)	Time heated (min.)	No. cultures with CPE	Interval (days) CPE first observed* 3–5	8–11	13–19	21–27	29	32–35
1	55	10	7	5	1	1			
		20	4	3					
		30	4	1	3				
		60	1	1					
	60	10				1			
	65	10							1†
		30					1		
		60					4		
2	60	10	1		1				
		60	1		1				
	65	10	3		1	1			1
		60	1						1
3	65	60	1		1				
4	75	60	1			1			

* In repeated titrations of unheated virus suspensions CPE was not noted after 6th day following inoculation.

† Questionable CPE on 32nd day.

original unheated stock suspension. A suspension of progeny virus was prepared consisting of fluid from a bottle culture of the first passage in monkey kidney cells. The culture was rinsed 5 times with Hanks' solution before and after inoculation and adsorption of virus. The viral suspension, therefore, probably contained less protein than the unheated suspension which was prepared in cultures that received fewer rinsings. After harvest, the suspension was centrifuged at 2000 rpm for 30 minutes at 4°C. Aliquots were heated at 55°C for various periods together with others of the stock Type II suspension from which it was originally derived. Assay of residual virus in each aliquot was carried out in amnion cell cultures. Results are presented in Fig. 1. It is evident that the progeny of the heated virus was more thermolabile than the parent or parents that had resisted 65°C, since a large proportion was destroyed at 55°C within 10 minutes. The greater overall thermoresistance of progeny virus however, as compared with that of the original unheated stock suspension is equally apparent. Both inactivation curves are inconsistent with a first order reaction.

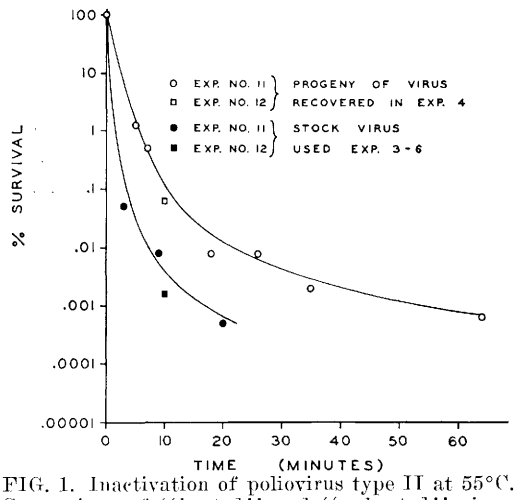

FIG. 1. Inactivation of poliovirus type II at 55°C. Comparison of "heated" and "unheated" virus.

To determine whether this difference in thermostability might be correlated with a difference in plaque morphology, dilute suspensions of progeny and stock viruses were applied to monolayers of amnion cells cultivated in small bottles. Nutrient agar was added. Plaques developed in each which were indistinguishable in respect to shape and size. Other characteristics that might differentiate them such as virulence for animals were not investigated.

Discussion. Two hypotheses may be presented that could account for the presence of these rare, highly thermoresistant forms. The first would regard them as extreme variants in a population in which there is a tendency by many individuals to diverge considerably from the mean thermoresistance. This has been

tacitly accepted in the foregoing portion of the paper. The second hypothesis would envision a primary injury of all units rendering them incapable of initiating infection. Thereafter resynthesis might rarely occur of components derived from different viral particles resulting in production of complete virus. Such a process would be analogous to multiplicity reactivation in bacteriophage. The only datum now available favoring this view is the observed delay in emergence of cytopathic change. At present, therefore, we incline to accept the first and simpler concept which is supported by the demonstrated decrease in rate of inactivation as heating is continued. It would seem, however, that on the basis of our findings (and they should be supplemented by results of experiments with clonal lines of virus) these highly resistant variants do not "breed true" in the sense that their progeny are equally resistant; instead, as one might expect, mean resistance of progeny is increased. This suggests that more than a single gene may determine, in association with environmental phenomena, thermoresistance of the progeny.

Factors to account for the difference between our results and those of previous investigators are not readily apparent. The concentration of extraneous protein in our preparations which might enhance thermostability was as low or lower than that in preparations examined by others. The pH was maintained at neutrality or slightly on the alkaline side where the stabilizing effect of cystine is not critically operative(11). It is possible that the higher concentrations of virus we employed together with replicate tests in a fairly large number of cell cultures of high sensitivity observed over long periods may be responsible for the discrepancy.

At first glance the data presented in Table I appear to suggest that thermoresistance might be somewhat greater at 65° than at 60°C. This paradoxical situation is probably to be attributed to the small number of determinations made. Survival of appreciable quantities of poliovirus under these conditions makes it doubtful whether pasteurization of milk and other food products as now carried out will assure completely inactivation of these agents.

Summary. Suspensions of Polioviruses Types I, II and III in medium 199 derived from infected monkey kidney cell cultures were not completely inactivated following exposure for 1 hour to temperatures of 60° and 65°C. In one instance infectious virus was demonstrated in a suspension of Poliovirus Type II after heating at 75°C for 1 hour. Progeny of virus surviving at this temperature exhibited increased thermoresistance.

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