STUDIES ON MICROORGANISMS IN SIMULATED ROOM ENVIRONMENTS

IV. The Effect of Survival on the Pathogenic Properties of Streptococci: Mouse Virulence[1]

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With a Statistical Appendix by Earle B. Phelps

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In a previous paper it was observed that streptococci which had settled onto the floor of an experimental chamber might survive in some instances for periods of time up to two weeks (Buchbinder and Phelps, 1941). The present paper is concerned solely with the effect of sojourn in the chamber on the property of mouse virulence of a strain of beta hemolytic streptococcus. The only recent reference in the literature to similar studies is in a paper by White (1936). She says "An experiment made by Dr. L. Colebrook has shown that hemolytic streptococci maintain their mouse virulence in dust undiminished for 25 days."

PLAN OF THE EXPERIMENTS

Description of the culture. The strain used in the study (090 of Lancefield; Aronson's streptococcus of Howie) although of uncertain origin has been widely used in this country and abroad. It belongs to Lancefield's Group B and is highly virulent for mice. Its pathogenic properties have recently been studied by Howie (1938).

The experimental chamber and method of inoculating cultures therein. This has been described in preceding papers (Buchbinder and Phelps, 1941; Phelps and Buchbinder, 1941).

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Recovery of cultures. Cultures were recovered in two different ways. The first method consisted of exposing sheep's blood agar plates to the bacteria settling from the air. The plates were placed in position by sliding a tray containing them onto the floor of the room through a small door in one wall. This method was adequate for obtaining samples until about thirty hours after spraying. It was modified in experiment I to obtain bacteria at intervals up to 104 hours by inserting a sterile broom through the door and sweeping the floor vigorously just after the dishes were inserted. The stirred up bacteria then settled into the dishes. The second method, used in experiments II and III, consisted of exposing sterile uncovered dishes to the air of the room from the inception of spraying until the day of removal. The sprayed organisms thus fell onto the glass bottoms of the dishes or alternatively onto sterile filter paper which had been placed therein. The dishes were joined in sets of six or more by means of strings attached to their under surfaces, so that they could be readily withdrawn through the door. After removal, plates were poured with sheep's blood agar and treated as described below.

Treatment of cultures and method of testing virulence. Cultures recovered from the room were incubated for 18 hours at 37°C. and grew out as individual colonies on the blood agar plates. Single colonies were then transferred to a tube of nutrient broth containing a drop of sheep's blood and incubated for 18 hours. The virulence of the recovered culture was tested in mice by inoculating them either with this first broth transfer or, in most instances, with a second subculture in broth.

In experiment I, in order to determine small changes in virulence, dilutions of each 18-hour broth culture were inoculated into ten mice, five receiving one ml. of a 1×10^{-7} dilution and five others one ml. of a 1×10^{-8} dilution. In experiment II, one ml. of the 1×10^{-8} dilution only was inoculated into each of five mice, while in experiment III the same dilution was inoculated into each of 10 mice. Inoculated mice were kept five to a cage and at least one dead mouse from each cage was examined for the presence of the infecting organism. All mice which had not died at the end of seven days were discarded and recorded as

surviving. The mean number of organisms inoculated was determined by plating one ml. amounts of the 1×10^{-6} dilution of each culture in duplicate or triplicate.

One strain of mice weighing between 18 and 20 grams, and obtained from one dealer, was used throughout.

A number of "unsprayed" cultures were used as controls. They were obtained anew for each experiment by streaking out on sheep's blood agar plates the culture used for spraying. Isolated colonies so obtained after 18 hours incubation were treated like the "sprayed" cultures thereafter. They were inoculated into mice at the same time as the recovered cultures.

EXPERIMENTAL

Experiment I. Mouse pathogenicity of the bacterial cultures was tested upon recovery from the dark room after sojourns of 8 to 104 hours. The experiment was run in three parts; intervals of exposure and the number of cultures obtained at each time interval are indicated in table 1. For analysis the data are pooled in table 2. It is seen that the strain is normally highly pathogenic for mice: 97.5 per cent of 160 mice injected with control cultures diluted 1×10^{-7} succumbed to an average dose of 17 organisms,[2] and 82 per cent of 160 mice, to an average dose of 1.7 organisms in the 1×10^{-8} dilution.

On the other hand 90 per cent of the mice injected with the 10^{-7} dilution and 71 per cent of those with the 10^{-8} dilution of the recovered cultures succumbed. The differences between the above figures and those for the corresponding controls are statistically significant. Thus it seems that a general lowering of the virulence of the recovered cultures has occurred. The statistical appraisal of the above data is given in the statistical appendix, Part A.

The evidence thus far indicates merely a general lowering of virulence. Whether this effect was spread over the whole series of recovered cultures or was due to a change among certain cultures, remains to be investigated. It is found that the 87 re-

[2] The term organism as employed refers to a single unit, generally a chain of several cells.

covered cultures may be separated arbitrarily into two groups, one composed of the least virulent 18 cultures, the other of the remaining 69. The latter group is now found to yield the same mean survival rate as the control group (2.5 per cent) and to be

TABLE 1

Distribution of 87 recovered cultures, in three series of experiments, by time spent in experimental chamber

TIME IN ROOM	NUMBER OF CULTURES		
	Part 1	Part 2	Part 3
hours			
1–8	9	10	
23–31	9		
30–32		10	
48–51		10	10
72			10
80			10
104			9
Total.....	18	30	39

TABLE 2

Comparison of mouse lethality of beta hemolytic streptococcus (strain 090, group B) before and after sojourn in a room from ½ to 4½ days

	INOCULATED WITH CONTROL CULTURES		INOCULATED WITH RECOVERED CULTURES	
	Dilution			
	1×10^{-7}	1×10^{-8}	1×10^{-7}	1×10^{-8}
Number of cultures.....	32	32	87	87
Number of mice injected.....	160	160	434	434
Average dose (Bacterial chains).....	17	1.7	18	1.8
Number "inoculated" (See Statistical Appendix).....	160	130 ±5	434	362 ±8
Number died.....	156	131	389	307
Per cent died (of injected).....	97.5	82	90	71
Per cent died (of "inoculated").....	97.5 ±1	100 ±4	90 ±6	85 ±7

statistically homogeneous. The 18 separated cultures give a mean survival rate of 41 per cent and are not homogeneous material. We believe that the virulence of these eighteen cultures, 21 per cent of the whole number, has been modified by sojourn

in the chamber, while that of the remaining 79 per cent is unmodified. (See Statistical Appendix, Part B.)

A group of cultures recovered after 48 hours in the chamber which showed this change in virulence were next titrated in mice to determine whether the virulence of larger doses of organisms had been altered as well. For this purpose, six recovered and two control cultures were each inoculated into five mice with decimal dilutions from 1×10^{-3} to 1×10^{-6}. The average dose in the 10^{-3} dilution was 220,000 for the control cultures and 226,000 for the recovered cultures. All but two of the 160 mice inoculated

TABLE 3

Distribution of 87 recovered cultures by time of sojourn in chamber and number of surviving mice from each set of five inoculated (10^{-7} dilution)

PART	TIME IN CHAMBER	NUMBER OF CULTURES YIELDING SURVIVAL OF					TOTAL	AVERAGE SURVIVAL
		0	1	2	3	4		
	hours							*per cent*
I	7–8	8	1	0	0	0	9	2.2
	23–31	6	3	0	0	0	9	6.6
II	7–8	9	1	0	0	0	10	2.0
	30–32	7	2	1	0	0	10	8.0
	48–51	1	2	2	2	3	10	48.0
III	48–51	4	3	3	0	0	10	18.0
	72	10	0	0	0	0	10	0.0
	80–96	9	1	0	0	0	10	2.0
	104–128	7	2	0	0	0	9	4.4

died within a week. The average time to death of all mice inoculated with the recovered cultures was 2.1 days and that for the control cultures 2.4 days. Thus, it appears that recovered cultures which seem to be less virulent than controls with small dosage, are not different when larger doses are inoculated. The facts are at least in harmony with the following hypothesis.

The modification of virulence noted is not only confined to a small proportion of the recovered cultures, but among the apparently modified cultures, to a proportion of the individuals in each culture. Thus, with a small inoculum only modified organisms

are introduced in many instances and a low death rate results. With larger doses of the same culture, unmodified individuals are certain to be introduced in each instance with a corresponding high death rate indistinguishable from that of the controls.

In an attempt to ascertain the permanence of the changes induced in the recovered cultures, redetermination of virulence of two recovered cultures was made three months after recovery. The 10^{-8} dilution of each culture was injected into 50 mice. It was found that 41 per cent of the mice survived (including those not inoculated) with a distribution which matches the binomial expansion for that per cent of survival. (See Statistical Appendix Part B, for method.) This result is significantly different from that of the 10^{-8} controls which was 18 per cent. However, when compared with the findings of the original group of altered cultures from which the above two cultures were selected, (10 cultures injected into a total of 50 mice with 72 per cent surviving), a significant increase in virulence was noted. A similar tendency of this strain to revert to original virulence has been noticed by Howie (1938) in cultures whose virulence has been lowered by growth in acriflavine broth.

An effort was also made to determine whether the apparent lessening of the mouse virulence of the above two cultures might be reflected by a change in their immunological behavior; namely, a quantitative decrease in their ability to provoke agglutinins in rabbits. The two recovered cultures and two control cultures were each used to inoculate a pair of rabbits, from a group of eight litter mates. The method of immunization was that of Pauli and Coburn (1937) and the killed vaccines used were standardized by means of a Petroff-Hauser chamber. The rabbit serums after immunization were tested for agglutination by the slide technique. It was found after a series of ten inoculations with heat killed vaccine that three of the four rabbits immunized with the control cultures had specific agglutinins whereas none of the four rabbits injected with the recovered cultures showed this response to immunization. After an additional series of five injections of both living and dead organisms it was found that all of the rabbits except one, treated with recovered culture, had produced agglu-

tinins. Continued immunization of the exceptional rabbit did not result in the production of agglutinins. A result similar to the above would occur by chance alone only once in nine trials. It may be concluded, therefore, within this limit of probability, that since seven of the eight rabbits were susceptible to immunization, the early appearance of agglutinins in those immunized with control cultures was an indication of some alteration in the protoplasm of the recovered cultures. Since this work was completed, Angevine (1939) has presented data of the same purport, namely, the delayed appearance of agglutinins in rabbits in response to a less virulent variant of a virulent streptococcus. Topley *et al.* (1937) have also reported similar findings with typhoid bacilli.

The next question asked was whether the changes in mouse virulence occurred after any particular time of exposure or whether they occurred in a haphazard manner. In table 3 all the recovered cultures are listed according to the number of mice surviving from each set of five inoculated with the 10^{-7} dilution. These data are also shown in figure 1, in which the percentage of survival of all mice for each time interval of each part of experiment I is plotted against the duration of sojourn of the cultures in the experimental room. It is seen that although the greatest percentage of survivors occurs in Part II and at 48 hours that this peak seems to be part of a general trend which reaches a peak at that time and then recedes.

To summarize the entire experiment briefly, of 87 cultures recovered from the experimental room at time intervals ranging from 8 to 104 hours, 69 (79 per cent) were unmodified, whereas 18 (21 per cent) had lost part of their virulence. No modified culture failed to kill at least one mouse of five at the 10^{-7} dilution. A probable decrease in the ability of their vaccines to produce agglutinins in rabbits was manifested by two cultures which showed a lowered mouse virulence. It was also found that most of the cultures showing lowered mouse virulence were recovered from the experimental chamber 48 hours after they had been sprayed therein. A possible explanation for this is given subsequently.

Experiment II. The purpose of this experiment was to deter-

mine the effect on mouse virulence (10^{-8} dilution) of longer sojourn in the experimental room, namely, $6\frac{1}{3}$ to 10 days. It is seen in table 4 that on the whole there was only a little change in virulence. However, the average survival rate of mice for the 29 recovered cultures was nine per cent whereas that for the 20

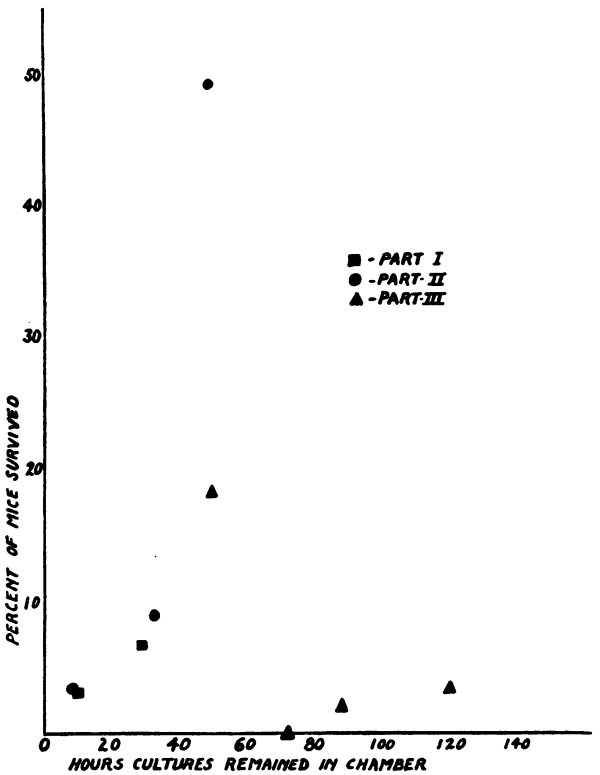

FIG. 1. RELATION BETWEEN MOUSE VIRULENCE AS MEASURED BY SURVIVAL RATE, AND TIME OF SOJOURN OF ORGANISM IN EXPERIMENTAL CHAMBER

control cultures was 5 per cent. The difference is 4.5 $\pm$4.2 per cent, which is not significant. The cultures responsible for this difference were disclosed when all of them were studied according to their time of exposure in the chamber. Thus, when the probable number of mice not inoculated was eliminated it was found that 8 per cent of the 6 day group (total of 30 mice), none in the

next three groups, 13 per cent in the 10 day group (15 mice), and 15 per cent of the 15 day group (5 mice), survived.

We feel justified in suggesting that the apparent modification in virulence is associated with the dying of the organism. At about 48 hours there is a maximum proportion of living, modified

TABLE 4

Comparison of mouse lethality of subcultures of a strain of beta hemolytic streptococcus before and after chamber sojourn from 6 to 10 days (10^{-8})

	INOCULATED WITH CONTROL CULTURES	INOCULATED WITH RECOVERED CULTURES
Total number of cultures	20	29
Total number of mice	100	145
Average dosage	2.1	1.9
Number died	84	112
Number "not inoculated"	12 ±3	22 ±4
Corrected per cent died	95.5 ±3.5	91 ±3

The total of 29 recovered cultures were exposed in room as follows: 6 for 151 hours; 6 for 168 hours; 6 for 192 hours; 7 for 215 hours; 3 for 240 hours; 1 for 316 hours.

TABLE 5

Comparison of mouse lethality of a strain of beta hemolytic streptococcus before and after sojourn in an artificially illuminated chamber from 1 to 8 days (10^{-8} dilution)

	INOCULATED WITH CONTROL CULTURES	INOCULATED WITH RECOVERED CULTURES
Total number of cultures	10	40
Total number of mice	99	395
Average dosage	3.4	3.3
Number died	87	340
Number "not inoculated"	4	16
Corrected per cent died	92 ±3	90 ±2

organisms. Later, up to five or six days, these tend to die more rapidly than the unmodified individuals. There is even some indication, after still longer periods of exposure, of a secondary rise in the proportion of modified strains continuing progressively as long as 15 days.

Experiment III. This experiment was undertaken to determine

the effect of exposure of the sprayed culture to artificial illumination. A fluorescent lamp (Buchbinder, Solowey and Phelps, 1941), was placed on each wall of the chamber so that the average illumination was about four to five foot-candles. Lights were on continuously and cultures were collected during the first eight days. The data as given in table 5 indicate that there was no significant difference (10^{-8} dilution) between the control group of cultures and the recovered group taken as a whole. The slight difference noted was due entirely to three of the 40 recovered cultures which yielded a corrected survival rate of 50 per cent.

DISCUSSION

The chief impression made by these experiments is that mouse virulence of the strain of streptococcus studied is remarkably stable. The virulence of the majority of cultures derived from cells exposed in the experimental room for periods as long as ten days were, within the limits of the technique, unaltered. The possible implication of this from the public health viewpoint is manifest. Are the disease-producing properties of streptococci and other bacteria similarly unchanged by exposure to the aerial environment of ordinary life? If the answer is affirmative it, together with the previously reported relatively long survival time of organisms in the experimental room (2 weeks), confirms the existence of a problem in sanitation hitherto neglected on the basis of an impression that bacteria cast into the air enjoy but a short life and even that coincident with attenuation.

Those cultures which appeared to have lost some virulence, when tested in doses up to about 20 organisms, were normally virulent when the criterion was a dose ten times that amount. Some evidence is adduced, furthermore, that the changes although statistically significant, were not permanent and that the ensuing alterations were in the direction of normal (increased) virulence. However, the occurrence of even small changes in virulence is of some interest. The fact that the maximum distribution by time in the darkened room of altered cultures was around 48 hours at the expense of both earlier and later periods is unexpected. One

possible explanation is that cultures modified in virulence have a higher death rate than the unmodified ones and so disappear sooner. The absence of any profound changes in mouse virulence of the recovered cultures in the experiment carried on in the presence of diffuse artificial light which, we have found, accelerated the normal death rate, might be explained on the basis of a more rapid death rate of modified cells under such adverse conditions.

SUMMARY

Experiments were undertaken to determine the fate as regards the property of mouse virulence of a strain of beta hemolytic streptococcus exposed to the environment of an experimental room. It was observed that the majority of cells exposed for time periods ranging from eight hours to about ten days were by a refined technique, unaltered in this property. The possible public health implications of this finding are discussed.

A small number of cells apparently underwent a minimal diminution in mouse virulence. This change occurred in a peculiar time distribution. It was found that the lowered virulence of these cells was apparently paralleled by a diminution in their antigenic activity in rabbits. However, it was noted that after the passage of several months the modified cultures tended to return to maximal virulence.

An interpretation is offered of the time distribution of these changes and their absence in cultures exposed to artificial illumination.

Statistical Appendix by Earle B. Phelps

Part A

In a bacterial suspension the distribution is not uniform. If there be, on the average, one organism per unit volume, certain unit volumes may contain two or more organisms, others only one and still others none. The respective probabilities of each of these and of other similar events are fully expressed as a func-

tion of the mean concentration, in the Poisson exponential series.[3] The mean concentrations of bacteria in the 1×10^{-7} and 1×10^{-8} dilutions of the thirty-two control cultures were used in the Poisson formula to determine the probability that each injected dose contained no organisms. The average of these thirty-two probabilities represents the expected proportion of animals injected but not inoculated from which we compute the number actually inoculated, line 4 of table 2. In the 1×10^{-7} dilution the number not inoculated is negligible. In the 1×10^{-8} dilution, however, 160 animals were injected of which 30 ± 5 probably received no organisms.

Apparently 2.5 per cent of the mice survived an average inoculation of 17 bacteria per mouse (1×10^{-7} dilution). This may represent the upper end of the curve of distribution of mouse resistance or the lower end of the curve of distribution of dose inoculated or both together. On the following reasoning we believe that it is wholly a matter of mouse resistance and not one of dosage.

On the basis of the Poisson series the 2.5 per cent of the inoculating doses having the fewest organisms contained from five to nine organisms per inoculation; about half of them containing just nine and half of the remainder just eight organisms. Apparently 2.5 per cent of the mice are resistant to at least this dosage. On the other hand, we have shown that 30 ± 5 animals injected with the 1×10^{-8} dilution probably received no organisms and by the same procedures it can be shown that an additional 50 ± 6 animals probably received only one organism.[4] There were 29 survivals. Within the statistical limit of accuracy it appears that, on the average, one organism inoculated will produce death

[3] The Poisson series is

$$e^{-x}\left(1 + X + \frac{X^2}{2!} + \frac{X^3}{3!} \cdots\right)$$

in which X is the mean number per unit volume and the successive terms are (1) the probabilities of their being 0, 1, 2, 3, $\cdots$ in a given unit volume sample, or (2) the expected proportion of such sample that will contain 0, 1, 2, 3, $\cdots$.

[4] The term organism as employed throughout this discussion refers to a single unit, generally a chain of several cells.

in most of the animals, but that about 2.5 per cent of the mice are resistant to inoculations as great as five to nine organisms. The numbers of organisms inoculated from the recovered cultures were essentially the same as for the controls. In the group of 434 mice receiving the 1×10^{-7} dilution all were inoculated, 389 died and 45 survived, or 10 ± 1 per cent. Statistically the 45 mice receiving the lower dosage might be expected to have been inoculated with the following number of organisms:

Organisms per dose	Number of mice
7	1
8	2
9	4
10	7
11	11
12	16
13	4
	45

It might be assumed that these 45 mice receiving the minimal dosage were also the 45 survivors, i.e., mice survived completely up to a dosage of about 13 organisms above which they succumbed completely.

As against this assumption there are the data on the 10^{-8} dilution which may be summarized as follows:

Average dose	1.8
Total mice injected	434
Uninoculated	72 ±8
Received just one organism	129 ±10
Received one or more organisms	362 ±8
Total mice survived	127
Uninoculated	72 ±8
Received one or more organisms	55 ±8
Survival rate (per cent)	
Based on 362 inoculated mice	15 ±5
Based on 129 inoculated with one organism	43 ±16

There were 362 animals presumably inoculated and of these 55 survived. On the basis of all inoculated animals the survival rate is 15 ± 5 per cent. But of these 362 inoculated animals 129

received just one organism, the remainder two or more. Were it to be assumed, as previously, that the survivors were all or largely contained within the low dosage group, it would yield a survival rate of 43 ±16 per cent. That is, one or more organisms per mouse is fatal 57 ±16 per cent of the time, whereas in the case of the 10^{-7} dilution the same assumption led to a complete survival of all mice inoculated with less than 13 organisms. The assumption therefore is untenable. The survivors are not found wholly or even largely concentrated among the low dosage animals.

In the recovered series, therefore, as in the control series, there is no significant difference between the survival rates whether the average dosage be 18 or 1.8. Survival depends upon the presence or absence of at least a single inoculated organism plus a variable mouse resistance.

There is, however, a real difference between the control and the recovered series which can be stated in terms of mean survival. Among the controls 160 + 160 − 30 = 290 animals were inoculated with one or more organisms and 4 survived or 1.4 ±0.7 per cent. In the recovered series, 434 + 434 − 72 = 796 animals were inoculated with one or more organisms and 45 + 55 = 100 survived, or 12.6 ± 1.9 per cent. This apparent change in virulence requires further analysis.

Part B

The reliability of a single set of five (or even 10) tests on one culture is not sufficient to determine alteration in virulence. It can be found, however, by the binomial expansion, that with an average survival of 2.5 per cent in the control series, the probability of 0, 1, or 2 survivors in a test of five is 0.88, 0.11 and 0.01. We would be unlikely to get two or more survivors and 88 per cent of the tests would show none. Making a comparison on the whole set of 32 cultures (control 10^{-7} dilution) we find

Mice surviving out of 5:	0	1	2	Total
Expected	28.1	3.5	0.4	32
Found	28	4	0	32

Thus, the distribution actually found suggests homogeneity among the control cultures, and tends to validate the value 2.5 per cent.

Applying this same test to the 87 recovered cultures in the 10^{-7} dilution, mean survival 10.4 per cent, the actual and expected series are:

Mice surviving out of 5:	0	1	2	3	Total
Expected	50	29	7	1	87
Found	61	15	6	5	87

This set of 87 cultures is clearly not of homogeneous character. Assuming the recovered cultures to be composed of a certain number of normal cultures (unmodified) and of others which have been modified, it is found that separating the 61 cultures that yielded no surviving mice together with 8 of the 15 that yielded one survivor, gives a set of 69 with a mean survival rate of 2.3 per cent and normally distributed. The remaining 18 cultures giving a mean survival of 41 per cent are distributed as follows:

Mice surviving out of 5:	0	1	2	3	4	5	Total
Expected	0	1	5	6	5	1	18
Found	0	7	6	2	3	0	18

The 18 cultures are not homogeneous.

Thus, the recovered cultures may be separated statistically into a group of 69 which have the same mean survival rates as the control series, and a normal distribution for homogeneous material, and 18 which have a mean survival rate of 41 per cent and a distribution which indicates that they are not of uniform character as regards virulence. This treatment of the data is not entirely justified but in view of all the facts presented it probably gives an approximate picture of the situation, namely a considerable loss of virulence on the part of only some (21 per cent) of the cultures, rather than a uniform loss on the part of all.

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