TOLERANCE TO BACTERIAL PYROGENS

I. Factors Influencing Its Development*

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Many different bacteria produce substances which, when administered parenterally to human beings or to experimental animals, cause elevation of the body temperature. These pyrogens are of some importance in clinical medicine, because their presence in materials injected for therapeutic purposes may cause severe pyrexial reactions in patients. On the other hand bacterial pyrogens are sometimes administered intentionally, to induce fever, as in the treatment of neurosyphilis by intravenous injections of typhoid vaccine.

Pyrogenic activity is a property shared by many different bacteria, although generally it is quantitatively greater in Gram-negative and bacillary forms than in Gram-positive or coccal forms. The pyrogenic fractions themselves are relatively stable to heat; ordinary sterilization in the autoclave does not inactivate them. Chemical investigation of these fractions has produced evidence indicating that they are complex carbohydrates (1–3). Some of these purified fractions have been antigenic while others have not. All such fractions are potent toxins, less than a milligram may cause death of a rabbit. Such knowledge as we have of their mode of injury indicates that there is widespread damage to capillaries and larger blood vessels (4). Additional evidence of this type of injury is found in the fact that the pyrogenic fractions of certain bacteria can elicit the Shwartzman phenomenon (5), and can cause hemorrhagic necrosis of mouse sarcoma (6).

The amount of purified bacterial pyrogen necessary to produce fever may be very small. As little as 0.5 microgram of a fraction from *Serratia marcescens* is sufficient to cause high fever in a rabbit (7). The mechanism by which this fever is produced has not been elucidated, but one significant feature is the latent period which is always observed between the time of the intravenous injection of the pyrogen and the beginning of the rise in body temperature. In human beings the time lag is usually between 45 and 90 minutes, while in rabbits it is usually 15 to 30 minutes. The existence of a latent period seems to indicate that the pyrogen does not act by a direct effect upon the temperature-regulating centers in the brain.

It is well known to clinicians that when patients are being given intravenous injections of typhoid vaccine as a method of fever therapy, the dose of vaccine has to be increased at successive treatments in order to bring about comparable temperature elevations. Some patients, after 8 or 10 bouts of fever, may re-

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quire as much as 250 ml. of typhoid vaccine for a single day's treatment (8). No satisfactory explanation of the mechanism of this remarkable tolerance has been given. The present communication describes a series of experiments done on rabbits to determine some of the factors which influence its development. A preliminary report has previously been published (9).

Materials and Methods

The pyrogenic materials employed were: (1) *Eberthella typhosa vaccine.*[1] This vaccine is ordinarily used for human immunization and fever therapy. The density of the bacterial suspension is approximately one billion organisms per ml. Microscopic examination shows few intact bacillary forms, the gross turbidity being due largely to cellular debris. (2) *S. marcescens vaccine.* The organism was obtained from the American Type Culture Collection. It was cultivated in tryptose phosphate broth at 37°C. for 4 days, then separated by centrifugation, suspended in physiologic salt solution, and killed by heating at 100°C. for 5 minutes. The density of the final suspension was approximately equal to that of the typhoid vaccine. (3) *Pseudomonas aeruginosa filtrate.* The organism was isolated in this laboratory, from the urine of a patient with pyelonephritis. A culture filtrate was prepared, following the procedure used by Welch *et al.* (10) in preparing a standard pyrogen for the First U. S. P. Collaborative Study of Pyrogens. (4) *Purified E. typhosa pyrogen* (2).[2] (5) *Purified S. marcescens pyrogen* (1).[3]

With each pyrogen a dose was determined which would elicit a marked febrile reaction but which would not cause death of the animal. In the case of typhoid vaccine, which was used in most of the experiments, the amount found to be suitable was 1 ml. of a 1:8 dilution. This is approximately 100 times the quantity necessary to produce a definite rise in body temperature in rabbits of this size. It has been given to more than 300 rabbits without a fatality. With the other 4 pyrogens, doses which elicited similar febrile responses were: *S. marcescens* vaccine, 1 ml. of 1:8 dilution in physiologic salt solution; *Ps. aeruginosa* filtrate, 0.5 ml.; purified *E. typhosa* pyrogen, 30 μg. in 1 ml. physiologic salt solution; purified *S. marcescens* pyrogen, 10 μg. in 1 ml. physiologic salt solution.

The physiologic salt solution used was tested at intervals, and was always pyrogen-free. Glassware, syringes, and needles were sterilized by dry heat at 170°C. for 2 hours. This is sufficient to inactivate any pyrogen present.

The rabbits used were males, weighing 2 to 3 kg. Several different breeds were employed, including New Zealand white, hare brown, and Chinchilla. The animals were housed in air-conditioned rooms in metal cages, at a temperature of 70–80°F. During experiments each rabbit was placed in a wooden stall, and secured by a head board. An opening in the floor of the stall permitted insertion and retention of a mercury thermometer without much disturbance of the rabbit. The rectal temperature was taken every 30 minutes. Animals whose temperatures were found to be higher than 103.6° were excluded from the test. Three readings were obtained before injecting a pyrogen, in order to establish a base line. No food or water was given during a test period. In order to avoid excessive fatigue, the animals were never kept in the stalls longer than 8 hours; this limited the observation period following pyrogen injection to 7 hours.

Temperature records of the rabbits were plotted on ¼ inch graph paper, using one vertical line for each degree Fahrenheit and one horizontal line for each half-hour of test.

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[1] Obtained from the Laboratories of the Georgia Department of Public Health.

[2] Obtained from Professor J. M. Nelson, Department of Chemistry, Columbia University.

[3] Obtained from Dr. M. J. Shear, National Cancer Institute, Bethesda, Maryland.

In order to obtain a numerical expression of both the height and duration of a fever, a "fever index" was calculated. This was done by taking as a base level the animal's temperature at the time of injecting the pyrogen, and measuring with a planimeter the area enclosed between this line and the course of the elevated temperature. When the temperature failed to return to the base level within the time limit, as occurred frequently with the first injection of pyrogen, in order to define an area for measuring the fever index a vertical line was drawn between the last temperature and the base line. The fever index is expressed directly in terms of vernier units of the planimeter. The instrument used was a Keuffel and Esser compensating planimeter No. 4236.

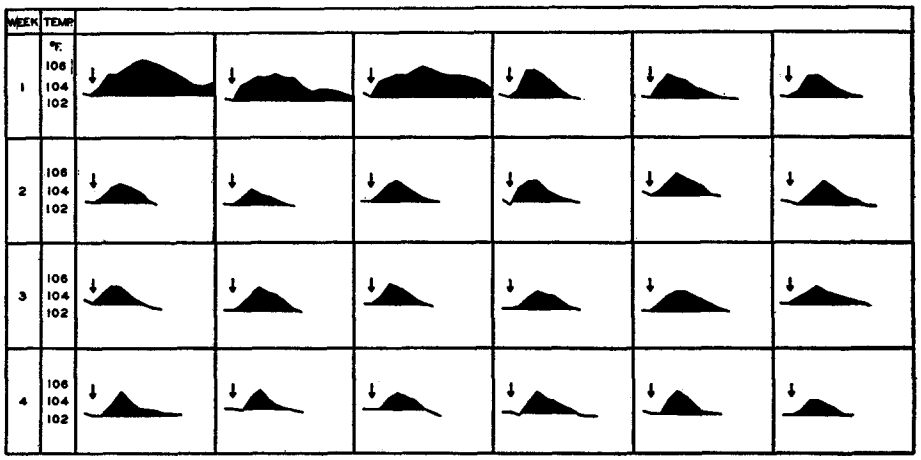

FIG. 1. Febrile reactions of a rabbit in response to daily intravenous injections of ⅛ ml. *E. typhosa* vaccine. The chart shows the characteristic marked diminution in febrile reaction at the end of the 1st week, with little change thereafter.

EXPERIMENTAL

Temperature Responses to Repeated Injections of the Same Dose of Pyrogen.— When animals were given daily injections of the same dose of bacterial pyrogen there was a characteristic pattern of response. The first injection usually caused a rise in body temperature 3–5°F., and the temperature seldom returned to the starting level within the experimental time limit of 7 hours. On the 2nd and 3rd days the febrile responses were nearly as severe as on the first injection, but from the 4th day onward a progressive reduction in pyrexial reaction was nearly always evident. Sometime during the 2nd week a state was reached at which a "minimal" febrile response was elicited, and after that the animal continued to react to each injection with about the same amount of fever; *i.e.*, a rise of 2–3°F., with return to the normal level in from 3 to 5 hours. Fig. 1 illustrates the successive fevers of one rabbit given a series of daily injections of ⅛ ml. of typhoid vaccine for a period of 4 weeks.

A collection of observations bearing on this subject is presented in Fig. 2. Here the fever indices of 85 animals are plotted according to the number of

daily injections of 1/8 ml. typhoid vaccine received. These comprise all of the observations obtained in the course of several different experiments done during this study, wherein no other modifying factor had been introduced. Temperature elevations were not measured every day in the various experiments; nevertheless it is apparent that the trend of response in this group corresponds to the preceding description.

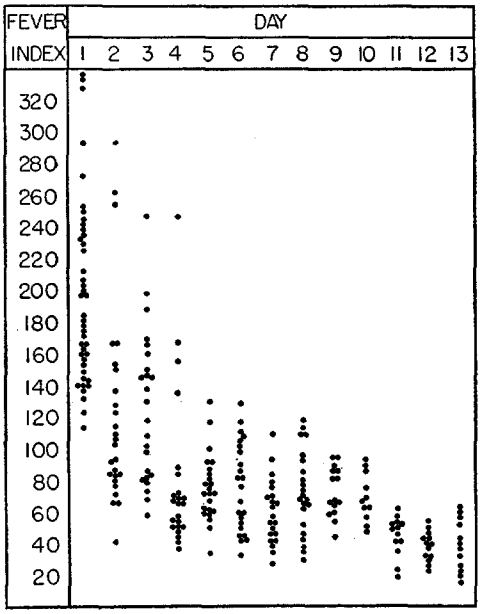

FIG. 2. Fever indices of 85 rabbits, plotted according to number of daily injections of 1/8 ml. *E. typhosa* vaccine. These data were obtained in the course of several different experiments. They show the trend to diminished febrile reaction which is observed when injections of pyrogen are given every day.

Repeated injections of *S. marcescens* vaccine, *Ps. aeruginosa* filtrate, or of the purified pyrogens of *E. typhosa* or *S. marcescens* brought out the same type of response, *i.e.* a progressive decline in febrile reaction during the 1st week or 10 days, thereafter a fairly constant "minimal" reaction to each injection.

Effect of Varying the Interval between Injections.—When injections of pyrogen were spaced at different intervals of time, it became apparent that the least febrile reaction was obtained when the pyrogenic material was injected every day. Animals injected only once or twice a week exhibited some diminution in their febrile responses, but this was not as marked as could be attained by daily injections of pyrogen. An example is shown in Fig. 3. There it will be observed that in a group of 4 rabbits injected with typhoid vaccine once a week for a period of 14 weeks, there was some diminution in the average fever

index, but that these animals, even after 14 weeks, had not developed a "minimal" response. They showed further reduction when the interval between doses was shortened to 1 day. Fig. 3 also shows the results in another group of 4 animals to which injections of typhoid vaccine were given twice a week for a period of 10 weeks. A series of 20 inoculations brought about a diminution in the febrile reactions, but again there was a further significant reduction when the interval between injections was shortened to 1 day.

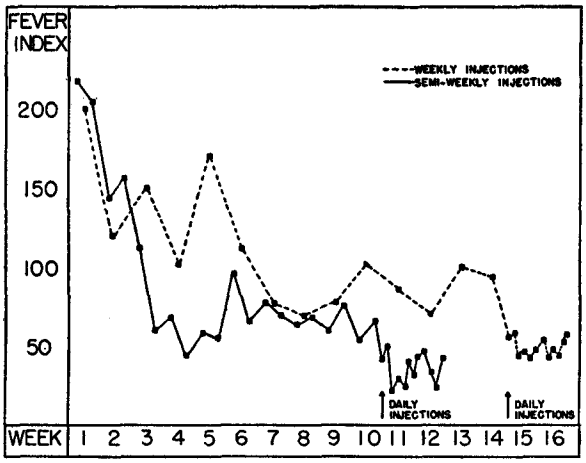

FIG. 3. Average fever indices in 2 groups of 4 rabbits. One of these received injections of ⅛ ml. *E. typhosa* vaccine once a week for 14 weeks, then a series of daily injections. The other group received injections twice a week for 10 weeks, then a series of daily injections. The results indicate that the tolerance to pyrogen becomes most highly developed when injections are given every day.

Loss of Tolerance after Rest Period.—When animals which had been rendered relatively unresponsive to a given dose of pyrogen were allowed to rest for some days and then retested, it was found that some increase in responsiveness had occurred.

For example, one group of 10 rabbits received ⅛ ml. of typhoid vaccine every day for 13 days. The average fever index for the 10 animals on the occasion of their first injection was 196. On the 13th day the average fever index for the same group was 39. Five of them were then rested for 8 days, and the remaining 5 for 22 days. On resuming the injections the fever index was found to have risen in the group rested 1 week to 85; whereas in those rested for 3 weeks the average fever index was 186. It appeared then, that after a rest period of 1 week, some, but not all, of the responsiveness had returned and that after a rest as long as 3 weeks the response was essentially the same as that of normal animals.

Lack of Correlation with the Titer of Specific Typhoid Agglutinins.—The level of serum agglutinins for the typhoid vaccine did not appear to be closely related to the febrile responses. In the preceding experiment the average titer

of antibodies in the rabbits which were rested for 1 week was 1:2560, while that of the rabbits rested for 3 weeks was 1:320. While there had been an appreciable reduction in titer at 3 weeks, the 4 animals still had considerable amounts of agglutinin in their blood, yet on reinjection of typhoid vaccine they gave a febrile response essentially like that of normal animals.

Effect of Reducing the Dose of Pyrogen.—Another experiment which demonstrated the rapid loss of responsiveness to pyrogens is illustrated in Fig. 4.

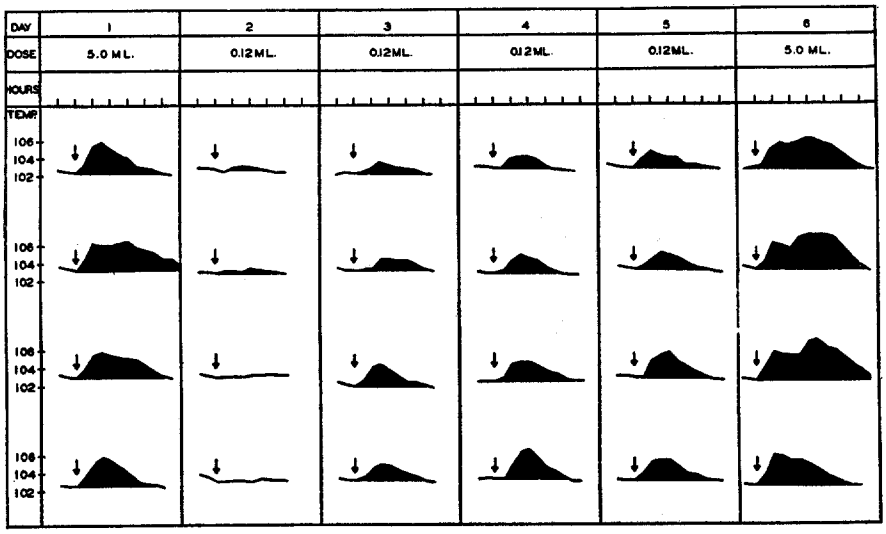

FIG. 4. Successive daily fevers in 4 rabbits during a period when the dosage of pyrogen was abruptly changed twice. During 2 weeks prior to the period charted each rabbit had been given increasing doses of *E. typhosa* vaccine, up to 5.0 ml. The left-hand column shows their febrile reactions to this large dose. On the 2nd day the dose was reduced to 1/8 ml. Note that there was little or no febrile reaction. Three more daily injections of 1/8 ml. caused higher fevers. On the 6th day a dose of 5.0 ml. was given again with the production of more fever than on the first charted day.

Four rabbits had received increasing doses of typhoid vaccine, beginning with 1/8 ml., and progressing to 5 ml. at the end of 2 weeks. Their temperature responses to this large dose are reproduced in the first column of this figure. On the following day, when the dose was reduced to only 1/8 ml., the animals reacted with little or no rise in temperature. The next day, injection of 1/8 ml. produced slight but definite fever in all of them, and thereafter the response to 1/8 ml. was similar to that of animals which had received a long series of daily inoculations with that quantity of pyrogen, as can be seen by comparison with Fig. 1. Finally, 5.0 ml. were given again. This induced somewhat more fever than on the first occasion, indicating that the reduced daily dose did not maintain a maximum tolerance.

This experiment was repeated on two occasions, with similar findings.

These results indicate that the animals are least responsive on the day after an injection, and that they become more responsive as early as the 2nd or 3rd day.

Specificity of the Tolerance for Different Bacterial Pyrogens.—When animals which had been rendered unresponsive to one bacterial pyrogen were tested with a different one, they were found to be somewhat tolerant of it as well.

For example, 5 rabbits were given 10 daily injections of ⅛ ml. of *S. marcescens* vaccine and then received ⅛ ml. of typhoid vaccine. The fever indices obtained were 98, 69, 98, 65, and 85. Comparison of these with first day responses of normal animals to that dose of typhoid vaccine, shown in Fig. 2, indicates that all were below the ordinary range. In another experiment, 8 rabbits received 10 daily injections of ½ ml. of *Ps. aeruginosa* filtrate, and then were given ⅛ ml. of typhoid vaccine. All showed a considerable reduction in febrile response as compared with normal animals; the fever indices being 76, 70, 74, 74, 71, 62, 66, and 70

Passive Transfer of Tolerance to Pyrogens.—An attempt was made to confer the unresponsive state by injection of serum from highly tolerant animals.

Three rabbits were given daily injections of typhoid vaccine in increasing amounts up to 5.0 ml. On the day following the last injection, when a high degree of tolerance would be expected, the 3 animals were bled by cardiac puncture, and their sera were pooled. This pooled serum was then given to 6 normal rabbits, in doses of 10 ml., intravenously. One hour later the recipient animals were given ⅛ ml. of typhoid vaccine. Six control rabbits were treated with normal rabbit serum and then with typhoid vaccine, in the same quantities. The febrile responses of all 12 rabbits fell within the range of ordinary responses (see first day responses, Fig. 2); and there was no appreciable difference in the fevers in the two groups.

Effect of Preventing Temperature Elevations during Development of the Tolerance.—

Eight rabbits were given daily injections of ⅛ ml. of typhoid vaccine. The febrile reactions to these injections were prevented by administering 0.6 gm. amidopyrine through a stomach tube, 1 hour previous to each injection of pyrogen. On the 8th day the premedication with amidopyrine was omitted. At this time, injection of the same dose of typhoid vaccine caused temperature elevations characteristic of animals tolerant to the pyrogen, the fever indices being 65, 54, 65, 106, 57, 103, 48, and 56. Reference to Fig. 2 will show that these were below the range of first day responses to this dose of typhoid vaccine.

It seems, then, that elevation of body temperature during the period of immunization is not essential to the development of tolerance to bacterial pyrogens.

Effect of Mechanically Induced Fever on Tolerance to Pyrogens.—An experiment was designed to determine the effect of a series of mechanically induced fevers on the temperature response to bacterial pyrogens.

For this purpose a fever cabinet similar to that used by Ellingson and Clark (11) was constructed. Four rabbits were given daily fevers for 8 days, their temperatures being raised rapidly to a level of 106–107.5°F., and maintained in that range for 2 hours each day. One rabbit died during this procedure, apparently of the effects of the pyrexia. On the day after the last of the mechanically induced fevers the 3 remaining animals were given injections of ⅛ ml. of typhoid vaccine. They reacted with high prolonged elevations, the fever indices being 191, 206, and 266.

The findings indicate that mechanically induced fever does not induce tolerance to bacterial pyrogens.

Behavior of Circulating Leucocytes during a Course of Injections.—In view of the marked fluctuations in circulating leucocytes which are known to result from intravenous injection of bacterial suspensions, leucocyte counts were made on a series of 6 rabbits throughout a 10 day course of injections of $\frac{1}{8}$ ml. of typhoid vaccine. As would be expected a leucopenia was found during the first hour after pyrogen injection, and this was followed by a leucocytosis in 4 to 8 hours. No striking alteration in this pattern of response was observed during the period of the injections except that the deviations in cell count tended to be smaller toward the end of the experimental period.

Effect of Repeated Injections of Pyrogens on the General Health.—Animals which received daily injections of pyrogens, in the quantities mentioned for periods of several weeks showed no sign of deterioration in general health. They tended to gain weight, their coats remained sleek, and there was no special tendency to develop intercurrent infections.

DISCUSSION

The experiments show clearly that rabbits given repeated injections of bacterial pyrogens become relatively unresponsive to the fever-promoting action of these materials. This is in accord with clinical observations on human beings undergoing fever therapy with typhoid vaccine. Such an alteration in temperature response of experimental animals has apparently not been observed by certain laboratory workers, since several recent reports of work with bacterial pyrogens indicate that animals have been used in tests repeatedly, sometimes daily, and that the possibility of alteration in reactivity has not been considered (12–14). Seibert, who was one of the first to demonstrate that pyrexial reactions to therapeutic intravenous injections were usually due to pyrogen contamination of the fluid (15), stated that in her experience rabbits never showed any indication of an immunizing or a sensitizing effect. This conclusion was supported later by Banks (16). A collaborative study of this matter was inaugurated by the Committee of Revision of the U. S. Pharmacopeia, in 1941 (17). A series of 3300 tests was made on 253 rabbits, a potent pyrogen (*Ps. aeruginosa* filtrate) being given to each animal twice weekly for 5 weeks. It was found that there was some lessening in the average height of the febrile reaction toward the end of the 5 week period. Our findings make it seem probable that those workers would have obtained more clear cut differences had the pyrogen injections been given daily. Another factor which explains in part the difference between our findings and those of other workers is that our calculations have included not only the height but also the duration of each fever. A characteristic of the immune response is the more rapid return of the temperature to the starting level (see Fig. 1).

The following evidence may be cited to indicate that the production of specific humoral antibodies may have little or no relation to the development of tolerance to pyrogens.

1. Animals lost their tolerance to typhoid pyrogen after a rest period of 3 weeks, that is to say at a time when specific agglutinins were still found in their sera.

2. The tolerance evidenced to one bacterial pyrogen obtained in some degree to pyrogens of bacteria not serologically related.

3. Passive transfer of the tolerance could not be accomplished.

4. The purified typhoid pyrogen, a carbohydrate material, which, by our tests, did not have antigenic properties, proved capable of inducing the same type of insusceptibility as vaccines of whole bacteria. Some observations of other workers support this evidence. Welch and associates showed that the pyrogenic fraction from *Ps. aeruginosa* was not antigenic (10). Favorite and Morgan studied the effects of a pyrogenic fraction from *E. typhosa* in human beings, and noted that "the titer of circulating antibody did not seem to be closely related to the development of tolerance to the toxicity of the antigen" (18).

SUMMARY

In a study of the febrile responses of rabbits to repeated intravenous injections of pyrogenic substances from *Eberthella typhosa, Serratia marcescens,* and *Pseudomonas aeruginosa,* the following observations were made:

1. A characteristic pattern of response to daily injections of the same dose of pyrogenic material was noted. This consisted of a progressive diminution in febrile response during the 1st week or 10 days, after which an animal responded to each injection with approximately the same degree of fever, even when the injections were continued for several weeks.

2. Animals given injections of the same amount of pyrogenic material at semiweekly or weekly intervals showed some diminution in febrile reaction but the alteration was less pronounced than that in animals injected every day.

3. Pyrogen tolerance appeared to be lost quickly. Animals allowed to rest for approximately 3 weeks reacted to readministration of pyrogen with fever comparable with that which occurred after the first injection.

4. By gradually increasing the size of the daily dose of pyrogen a tolerance could be established such that a reduced, but still considerable, amount of pyrogen caused no fever whatever.

5. Rabbits that had been injected with *S. marcescens* or *Ps. aeruginosa* pyrogens showed a diminished febrile response to *E. typhosa* vaccine.

6. Passive transfer of the unresponsiveness to pyrogens could not be demonstrated.

7. Prevention of temperature elevations during the course of immunization

by use of an antipyretic drug did not interfere with the development of tolerance to pyrogens.

8. A series of mechanically induced bouts of fever did not reduce the responsiveness to bacterial pyrogens.

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