BACTERIAL PROTOPLASTS

II. BACTERIOPHAGE MULTIPLICATION IN PROTOPLASTS OF SENSITIVE AND LYSOGENIC STRAINS OF *BACILLUS MEGATERIUM*

by

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It is interesting to speculate as to which structures of the bacterial cell can be removed without impairing specific functions. Recently it has become possible to dissolve the cell wall of *Bacillus megaterium* under conditions of controlled enzymic digestion with lysozyme. When this is done in the presence of a suitable concentration of sucrose, spherical protoplasts are formed—two, three or four from each rod of the original bacillus (WEIBULL[1]). It occurred to one of us (M.R.J.S.) that protoplasts may retain sufficient structural organization to support the growth of bacteriophages. Phage active against the intact organism is not adsorbed by protoplasts[1] as might have been anticipated from the fact that the primary interaction between host and virus probably occurs as a specific reaction of the phage tail with the bacterial cell wall. Also SALTON AND STENT[2] have shown that isolated cell walls of *B. megaterium* will react with appropriate bacteriophages.

There is good evidence for at least some bacteria/bacteriophage systems, that, after adsorption of the phage on the cell wall, the deoxyribonucleic acid (DNA) of the phage passes into the host leaving the protein coat outside. The bulk of this phage protein can be removed without impairing the ability of the host/phage DNA system to continue the normal course of intracellular phage development, lysis of the bacteria and liberation of many mature phages from each organism (LURIA[3]). Knowing that much of the physiological integrity of intact cells is still maintained in protoplasts and that the cell wall is not essential for protein and nucleic acid synthesis (McQUILLEN[4]), it was anticipated that bacteriophage formation might be possible in bacteria infected with virulent phage prior to removal of their walls by lysozyme.

Bacteriophage synthesis can also be studied in the lysogenic system which does not involve the specific structural relationship concerned in infection. Lysogenic strains of bacteria are believed to carry prophage as a genetic component (LWOFF[5]). In such cultures "spontaneous" phage production often arises by what is thought to be lysis of a small proportion of cells in which prophage has developed into many mature phages. Treatment of growing cultures with inducing agents such as ultra-violet light, certain reducing substances, or hydrogen peroxide, may cause most or all of the bacteria to produce mature phages and eventually lyse. In many respects the sequence of events

References p. 472.

after induction of a lysogenic culture resembles that after infection of a non-lysogenic culture with virulent phage.

Protoplasts have been shown to be capable of supporting the growth of virulent bacteriophages in the lytic system and also temperate bacteriophages in the lysogenic system.

MATERIALS AND METHODS

Bacterial cultures and phage used in this investigation were: *Bacillus megaterium* strain KM (NORTHROP[6]) sensitive to bacteriophage strain C (described by GRATIA[7]) and the lysogenic *Bacillus megaterium* 899(1) (DEN DOOREN-DE JONG[8]).

Growth medium. The organisms were grown on a synthetic medium, C/G, with aeration at 30° (MCQUILLEN[4]).

Bacteriophage stocks. High titre stocks of C phage were prepared in 5% Bacto peptone broth by harvesting phage from confluently lysed *B. megaterium* KM plated in 5% peptone, 0.7% agar layered on 5% peptone, 2% agar. The soft-agar layer was dispersed in 5% peptone broth, centrifuged and the supernatant stored at 4° C after filtration through a sintered glass bacterial filter.

Bacteriophage assay. Both virulent phage C and temperate phage from lysogenic *B. megaterium* 899(1) were assayed with *B. megaterium* KM which was grown overnight at 30° C on a 2% peptone agar slope and washed off in 50 ml 5% peptone broth. To 0.9 ml of bacterial suspension was added 0.1 ml of a sample for titration appropriately diluted with 5% peptone. 0.05 ml of phage/bacteria suspension was evenly spread on the surface of a medium containing 5% peptone, 2% agar. The numbers of plaques were recorded after *ca.* 12 h incubation at 30° C. Phage assays by this method were generally higher than by the conventional soft-agar layer method[9].

Inducing agent. 100 vol. hydrogen peroxide (Analar reagent) was suitably diluted so that small volumes could be added to cultures for induction experiments.

EXPERIMENTS AND RESULTS

Virulent bacteriophage system

Preliminary experiments with peptone-grown cells of *B. megaterium* KM showed that the burst-size for the intact organisms infected in peptone was about 400. Aerated cultures of infected cells were observed under phase contrast during the period of lysis and it was interesting to find that a high proportion of the organisms still left in the viscous medium were indistinguishable from protoplasts formed by action of lysozyme rather than being typical chains of bacilli. The rocking tube procedure[4] was equally effective for intact cell/phage experiments but a satisfactory method for handling protoplasts in peptone was not found. It was, therefore, decided to work with organisms which had been grown in the synthetic medium C/G as they were known to give stable protoplasts capable of synthesising protein and nucleic acid in C/G supplemented with sucrose *i.e.* C/G/S (MCQUILLEN[4]).

B. megaterium grown and incubated with bacteriophage in C/G did not give rise to an increase in phage. This may have been due to phage inactivation or unsuitability of the medium for adsorption or phage development. The first two of these possibilities were investigated. Samples of high titre phage stocks in peptone were diluted 10^8 with either peptone or C/G and incubated at 30° C for periods up to an hour. Titrations gave essentially identical results in all cases showing that the medium C/G did not inactivate the bacteriophage. On the other hand, when a mixture of phages and cells in C/G was plated after 5 min at room temperature and then centrifuged and titrations made on the supernatant and the resuspended pellet, over 95% of the phages were recovered in the supernatant. This implied that C/G was not satisfactory as an adsorption medium.

The best approach, therefore, seemed to be to use C/G-grown cells, infect in peptone

References p. 472.

and then carry out the incubations in C/G/S. The production of bacteriophages could then be followed in parallel with and without conversion of the cells to protoplasts by the addition of lysozyme. Both high and low multiplicities of infection were used.

High multiplicity infection

An overnight culture of *B. megaterium* in C/G was harvested, washed with peptone and resuspended in 1/10 volume of peptone. 0.6 ml was mixed with 0.4 ml of phage stock (titre $2 \cdot 10^{10}$/ml) and 0.1 ml samples removed for plating immediately and after 15 min at room temperature. The residual suspension was diluted to 10 ml with peptone, spun down, rinsed with C/G/S and resuspended in 8 ml of C/G/S. 1.0 ml samples were put into each of three tubes labelled KM, P and L. 0.1 ml lysozyme solution (1 mg/ml) was added to P and L and 0.1 ml water to KM. After 15 min protoplast formation was complete in P and L and the contents of all three tubes were diluted tenfold —KM and P with C/G/S and L with C/G. The latter tube was also shaken to ensure lysis of the protoplasts. After removing 0.1 ml samples for plating, the three tubes containing intact cells (KM), protoplasts (P) and lysed protoplasts (L) respectively, were closed with ground-glass stoppers and put on the rocking platform at 30° C. Further 0.1 ml samples were removed at intervals during 3 h. The lysed protoplast tube (L) was used as a control in case any intact cells survived lysozyme treatment. Fig. 1 gives a plot of the development of phages in the three systems.

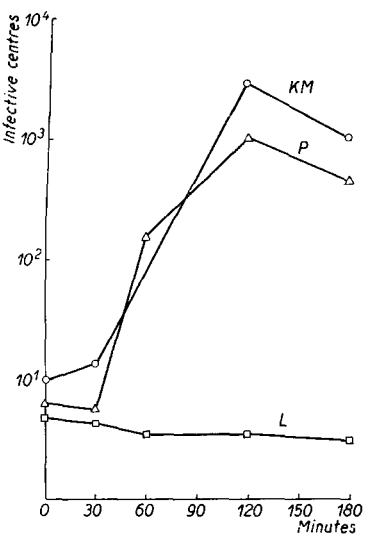

Fig. 1. Phage production after high multiplicity infection of *B. megaterium* KM with phage C. Intact cells (KM), protoplasts (P) in C/G/S, lysed protoplasts (L) in C/G, incubated by rocking at 30° C. Whole cells were infected in 5% peptone before washing and conversion to protoplasts.

The titre in the intact cell preparation increased about 300-fold; that in the lysed protoplast preparation remained unchanged; while with the protoplasts the phage count rose to about 1/3 that found with intact cells. This increase was coincident with bursting of the protoplasts, thus showing a close relationship to the pattern followed by infected whole organisms.

These experiments demonstrated unequivocally that bacteriophages could multiply in the sub-cellular host system of protoplasts. However, there were certain disadvantages in working with high multiplicities of infection—firstly, the recovery of input phages was always low since one cell infected with many bacteriophages would give rise to only one plaque; and secondly, there was little evidence of an "eclipse period"[3] (see DISCUSSION). Further experiments were therefore carried out with much lower multiplicities of infection.

Low multiplicity infection

The procedure was similar to that described above except that phage/cell ratios of much less than unity were used. Titration of the supernatant after infection showed that 40–90% of the added phage was adsorbed in 5–15 min and 50–80% of the input phages were recovered as infective centres from the supernatant plus the time zero sample of intact cells. Again the three systems were studied—intact cells, protoplasts

References p. 472.

and lysed protoplasts (Fig. 2 A and B). In these experiments the "eclipse period" was well evident—in Fig. 2 A the titres for P and L, the protoplast preparations, at zero time were only 1.3% of that for the intact organisms, KM. After a latent period of 15–30 min the titres rose in all cases, the burst size for the normal bacteria being 300–400. Lysed protoplasts showed some increase but the final phage yield was only about 1% of that from unbroken protoplasts, whereas that for the latter was up to 25% of that from intact cells.

Bacteriophage formation in a lysogenic system

As a preliminary to investigating the ability of protoplasts of the lysogenic *B. megaterium* 899(1) to produce bacteriophage, it was necessary to determine the suitability of

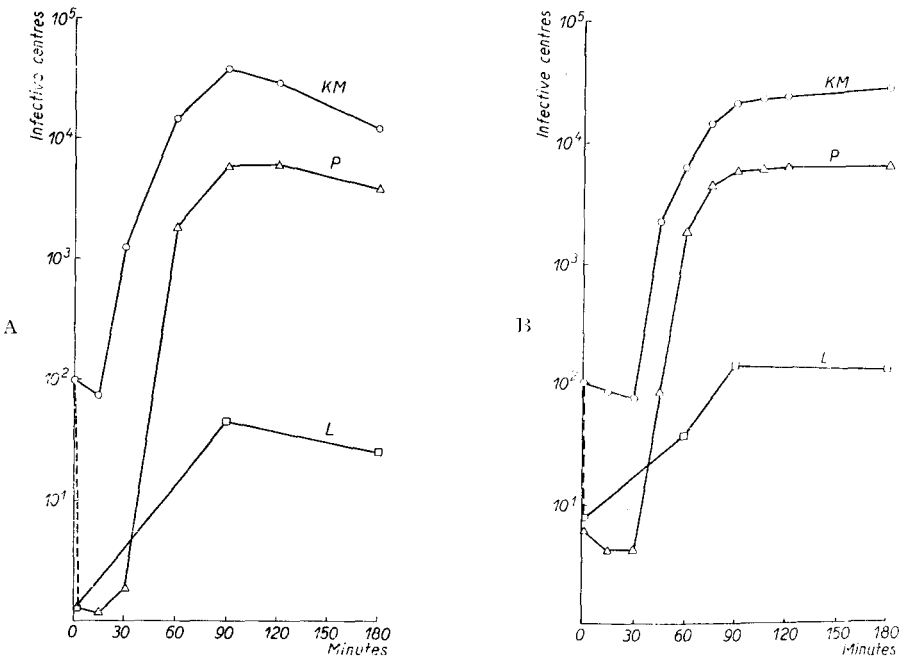

Fig. 2 A and B. Conditions as for Fig. 1 except that multiplicity of infection was less than 1.

media for the maintenance of lysogeny and for phage production in response to inducing agents. It is now well known that *B. megaterium* loses its lysogeny when subcultured on certain media (LWOFF[5]; CLARKE[10]; DE CARLO, SARLES AND KNIGHT[11]) and that not all media are suitable for induction experiments. For our experiments, a medium containing 2% Bacto tryptone and 0.6% Bacto yeast extract was initially tried as a growth and induction medium. The addition of $M/15{,}000$ hydrogen peroxide to exponentially growing cells in this medium resulted in successful induction. However, after several successive subcultivations in the medium, the organism showed a progressive decline in response to induction with hydrogen peroxide and after nine transfers the addition of hydrogen peroxide (final concentrations ranging from $M/5{,}000$ to $M/30{,}000$) did not bring about induction of the culture.

Loss of lysogeny on continued subcultivation in synthetic media both in the presence and absence of Ca-binding substances has been reported (LWOFF[5]; CLARKE[10]). While

References p. 472.

cultures of *B. megaterium* maintained on the synthetic medium C/G could not be induced with hydrogen peroxide when growing exponentially in this medium, the continued lysogeny of the organism was demonstrable on induction with hydrogen peroxide following a single transfer into the tryptone-yeast extract broth. The lysogenic nature of *B. megaterium*, as indicated by the "spontaneous" phage production and response to induction with hydrogen peroxide in an "apt" medium[5], has been maintained during 80 subcultivations on the C/G medium (McQUILLEN AND SALTON[12]).

Although induction under these conditions was regularly possible in the complex medium, it was felt that a defined induction medium would have certain advantages. The addition of hydrogen peroxide to exponentially growing cultures in C/G supplemented with a mixture of 17 amino-acids (*i.e.* C/G/17AA—see[12]) brought about induction—lysis of the cells + phage titres of *ca.* 10^{10} infective centres/ml. The presence of sucrose (7.5% w/v final concentration) which is necessary for the stability of the protoplasts did not alter the suitability of the C/G/17AA for induction experiments.

The media developed in these experiments satisfied requirements for the two prerequisites, maintenance of lysogeny and suitability for induction, and in addition had the great advantage of being simple and chemically defined.

For studies of the multiplication of bacteriophage in protoplasts of lysogenic *B. megaterium*, the organism grown overnight at 30° C with aeration in the C/G medium was inoculated into C/G/17AA/Sucrose. The development of the bacteria was then followed turbidimetrically and all experiments were performed on exponentially growing cultures which had attained an optical density of *ca.* 0.25 (1″ tubes read at a wavelength of 450 mμ in a Unicam spectrophotometer, model 350 D.G.). With lysogenic *B. megaterium* grown in this way two types of experiment were attempted—induction of cultures with hydrogen peroxide prior to conversion of cells to protoplasts with lysozyme, and induction by hydrogen peroxide after formation of protoplasts.

Induction of cells prior to conversion to protoplasts

Cultures grown in C/G/17AA/S as described above were divided into two equal parts and to one part, distilled water was added as a control, to the other part hydrogen peroxide (final concentration $M/15{,}000$). Aeration was continued in both cultures for 15 min at 30° C. 1.0 ml samples of the uninduced culture were pipetted into each of three tubes labelled 899, P and L and a similar set of tubes was prepared with 1.0 ml samples from the induced culture. 0.1 ml lysozyme solution (1 mg/ml) was added to P and L tubes of each set and 0.1 ml water to 899 of each set. Protoplast formation was complete in 15 min in P and L tubes of each culture and the contents of all six tubes were diluted tenfold—899 and P of each set with C/G/17AA/S and L of each set with C/G/17AA. To ensure the complete rupture of protoplasts the latter pair of tubes were vigorously shaken. All six tubes were sampled immediately for initial phage assays and then closed with ground-glass stoppers prior to incubation on the rocking platform at 30° C. Further 0.1 ml samples were taken at intervals throughout the course of the experiment. As with the virulent phage system, the lysed protoplast tubes (L) were included in the experiments so that the contribution by intact bacteria that might have escaped treatment with lysozyme could be assessed. The results of two typical experiments presented in Fig. 3 A and B illustrate the course of bacteriophage production in intact cells (899), protoplasts (P) and lysed protoplasts (L) of uninduced and induced cultures of *B. megaterium* 899(1). It was of interest to note that the rates of phage

References p. 472.

production in protoplasts formed from induced and uninduced cells compared most favourably with the rates for corresponding intact organisms. Although the protoplasts from induced cells showed as much as a 100–1,000-fold increase in phage titre, the maximum yield was only 1/7–1/60 of that of induced control cells.

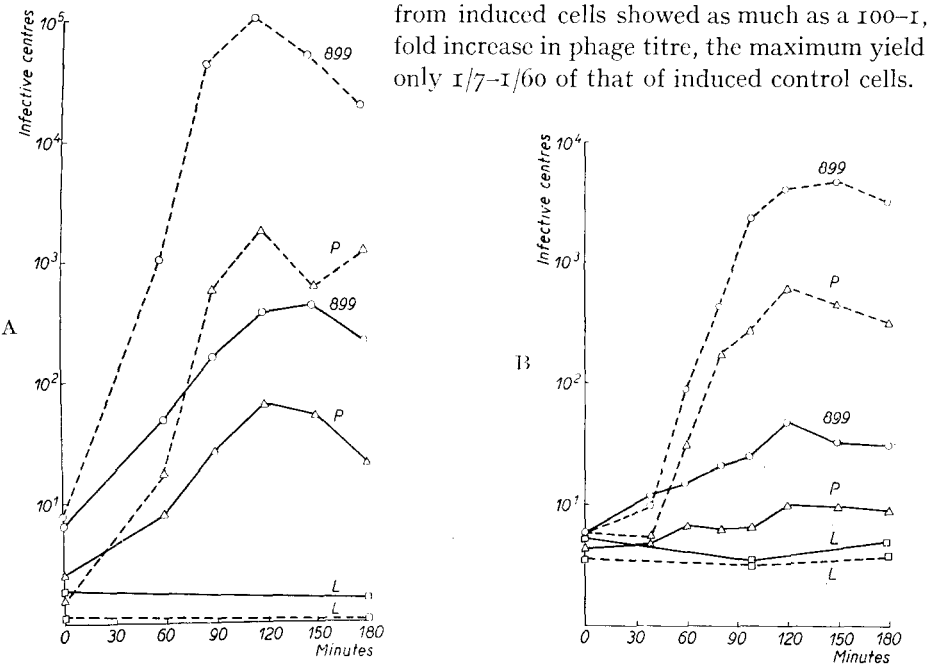

Fig. 3A and B. Bacteriophage production in intact cells (899), protoplasts (P) and lysed protoplasts (L) of *B. megaterium* S99(1) with and without induction by M/15,000 H_2O_2. Cells growing exponentially in C/G/S/17AA induced 15 min before conversion to protoplasts. —— Uninduced; ------- Induced.

Induction after conversion to protoplasts

Similar procedures to those described above were adopted when attempts were made to induce protoplasts directly. The numbers of infective centres in protoplast suspensions and in lysed protoplasts, with and without hydrogen peroxide (*M*/15,000) were determined during the course of three hours incubation. The addition of inducing agent did not result in an increase in phage titre above the level of the control protoplasts. In order to determine whether protoplasts required conditions different from those suitable for induction of intact bacteria, further experiments were performed with hydrogen peroxide concentrations varying from *M*/6,000–*M*/60,000. Over this range, the addition of hydrogen peroxide to suspensions of protoplasts did not result in direct induction.

DISCUSSION

We are happy to record that BRENNER AND STENT have independently demonstrated bacteriophage formation in protoplasts and we mutually agreed to submit results of investigations simultaneously.

The results obtained with the two systems described in this paper—the development of bacteriophages in protoplasts of sensitive and lysogenic strains of *B. megaterium*

References p. 472.

—demonstrate that removal of the cell wall of an organism, if carried out under carefully controlled conditions, leaves a sub-cellular structure still capable of performing intricate functions. In the case of the virulent system, protoplasts were able to synthesize new phage material and assemble it into mature bacteriophage while being incubated in a glucose/sucrose/NH_3 medium. To our great surprise the rate at which this occurred and the extent, *i.e.* the burst size, were not substantially different from those in normal cells having regard for the fact that some protoplasts inevitably lysed during the manipulations. That this ability was associated with protoplasts rather than with residual intact cells which had escaped lysozyme action, is shown by the unchanged phage titres of protoplast preparations lysed by shaking in diluted sucrose media before incubation (Fig. 1).

Current theories of phage multiplication suggest that after attachment of the phage particle to the bacterial cell wall, the DNA alone enters the cell and that for the first part of the latent period no mature phage can be recovered even after rupture of the bacterial cell. Owing to the fragility of protoplasts, these lyse during plating and until mature phages have been newly formed, low titres would be expected as an indication of the "eclipse period". Since intact cells do not lyse, maturation of phage can occur after plating so that the "eclipse period" can only be demonstrated when bacteria are intentionally disrupted before plating. Protoplast experiments with a high multiplicity of infection showed little or no "eclipse" since titres at the beginning of incubation were only slightly lower than those for the corresponding control (Fig. 1). The carry-over of a high proportion of free phages or phages reversibly bound on wall fragments might account for this finding and such an effect would be exaggerated by high multiplicity of infection.

On the other hand, when phage/cell ratios of less than unity were used, the "eclipse period" was quite apparent. In Fig. 2A the titres from the protoplast preparation during the first 30 min of incubation were only 1–2% of those for the intact cells. A 1,000-fold increase in phages occurred in the next half hour and eventually the yield from protoplasts was about 1/5 of that from normal cells. In other experiments even better performance was obtained.

With the knowledge that protoplasts supported the multiplication of virulent bacteriophage it was not surprising to find that protoplasts formed from induced, lysogenic bacteria also preserved sufficient structural integrity to synthesize mature phage. Virus formation in protoplasts and whole organisms from induced cultures occurred more rapidly than in the corresponding uninduced systems. But a parallel relationship usually exists between the rates of phage multiplication in protoplasts and intact cells in each case (Fig. 3 A and B). The observed course of phage production in induction experiments resembles that in the virulent phage experiments, again emphasizing the similarity between the transition from prophage–gonophage–mature phage in the lysogenic system and infecting phage–gonophage–mature phage in the lytic system (see LWOFF'S glossary[5]).

The extent of bacteriophage production was always less in protoplasts than in bacteria in this lysogenic system. This difference is not surprising considering that bacilli may reproduce during the course of the experiment (LWOFF[13]; CLARKE AND COWLES[14]). Although protoplasts cannot apparently divide they may nevertheless give a maximum phage yield as high as 1/7 of that of intact organisms. Individual rates and phage yields have varied from one experiment to another but in view of the complexity of the physiological conditions governing "aptitude" such differences were not unexpected.

References p. 472.

Repeated attempts to carry out direct induction of protoplasts have so far failed. The reasons for this failure can only be surmised. At present there is no satisfactory explanation of the mechanism whereby inducing agents initiate the transition from prophage to the vegetative unit gonophage, even in the intact cell. It may be that the interaction with inducing agent is different in protoplasts or that the nature or location of the prophage is in some way altered, for although protoplasts retain remarkable synthetic abilities[4], the full extent of the change resulting from transformation of rod-shaped cells to spherical protoplasts is far from being defined in biochemical terms.

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SUMMARY

1. Protoplasts of *Bacillus megaterium* KM are capable of supporting the growth of bacteriophage C if cells are infected prior to lysozyme treatment.

2. Protoplasts of lysogenic *Bacillus megaterium* 899(1) allow of development of the temperate bacteriophage if cells are treated with inducing agent before the walls are removed.

3. The rates and extent of phage synthesis in each system are comparable with those in bacteria which have not had their cell walls digested with lysozyme.

RÉSUMÉ

1. Les protoplastes de *Bacillus megaterium* KM sont capables de supporter la croissance du bactériophage C si les cellules ont été infectées avant le traitement par le lysozyme.

2. Les protoplastes de *Bacillus megaterium* 899 (1) lysogène permettent le développement du bactériophage tempéré si les cellules sont traitées avec l'agent inducteur avant que les parois cellulaires soient éliminées.

3. La vitesse de la synthèse de bactériophage et la mesure dans laquelle cette synthèse a lieu dans les deux systèmes sont comparables à celles que l'on trouve dans des bactéries dont les parois cellulaires n'ont pas été digérées par le lysozyme.

ZUSAMMENFASSUNG

1. Protoplaste von *Bacillus megaterium* KM sind imstande, das Wachstum der Bakteriophagen C zu gestatten, falls die Zellen vor der Lysozymbehandlung infiziert werden.

2. Protoplaste des lysogenen *Bacillus megaterium* 899 (1) gestatten die Entwicklung des gemässigten Bakteriophagen, wenn die Zellen, noch vor der Zerstörung der Wände, mit dem Induktionsmittel behandelt werden.

3. Geschwindigkeit und Umfang der Phagensynthese in beiden Systemen ähneln denjenigen, welche von Bakterien aufgewiesen werden, deren Zellwände nicht durch Lysozym zerstört wurden.

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