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Morphological manifestations of freezing and thawing injury in bacteriophage T4Bo

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SUMMARY

Electron microscopic observation of negatively stained preparations of frozen and thawed suspensions of T4Bo phage clearly separated the morphological changes produced by low-temperature salt denaturation from those produced by eutectic phase changes. Salt denaturation caused contraction of tail sheaths. Eutectic phase changes appeared to cause two separate lesions. Firstly the tail sheath was disjointed 18–22 nm. below the collar and the tail core was disjointed at 40–60 nm. below the collar, giving rise to separated heads with a small tail remnant, and separated tails in which the sheath remarkably remained in its extended form. Secondly, tears were seen in the head membranes of particles with collapsed empty heads. In all the experiments the percentage of normal phage particles counted electron-microscopically was close to the percentage of viable phage as determined by plaque assay.

INTRODUCTION

Three distinct mechanisms have been proposed to account for the inactivation of T4 phage and T4Bo (osmotic-shock resistant) phage during freezing and thawing, and eutectic phase change injury (Leibo & Mazur, 1969; Steele, Davies & Greaves, 1969*a*, *b*). The nature of T4 phage inactivation by osmotic shock is well understood and it has been shown both chemically (Hershey & Chase, 1952) and electron-microscopically (Kleinschmitt, Lang, Jacherts & Zahn, 1962) that DNA is released from the heads of osmotically-shocked T-even phage. The ethidium bromide fluorometric technique for estimating DNA (Le Pecq & Paoletti, 1966) has been used to show that DNA is quantitatively released from T4 phage osmotically shocked by slow freezing to temperatures between 0° C and −20° C. followed by rapid thawing (Steele, 1972). Salt denaturation causes non-osmotic inactivation of T4 and T4Bo phage above the eutectic temperature of the salt or salt mixture present in the suspending medium. The order of effectiveness of different salts in causing inactivation of stabilization of these phage follows the classical Hofmeister series. Eutectic phase change injury (eutectic injury) occurs when these phage are frozen to temperatures below the eutectic temperature of the suspending medium. T4Bo phage which survive the eutectic phase change of sodium chloride are latently injured (as manifest by hypersensitivity to a second eutectic phase change or ultrasonic vibration) which resolves if the suspensions are incubated

at 37° C. for 3 hr., and furthermore can be inactivated below the eutectic temperature by rapid cooling to −90° C. followed by slow rewarming (Steele, 1976).

The purpose of the present experiments was to examine frozen and thawed suspensions of T4Bo phage electron-microscopically to determine whether inactivation during freezing and thawing gives rise to distinctive morphological changes in phage particles and, if present, to what extent such changes can be correlated with the physicochemical events occurring during freezing and thawing.

METHODS

The methods of phage preparation, purification and titre determination were the same as those described previously (Steele *et al.* 1969*a*). Purified phage stocks of approximately 2×10^{12} p.f.u./ml. were suspended in 0·01 M phosphate buffer (KH_2PO_4-Na_2HPO_2, pH7) at 4° C.

Experimental procedures

T4Bo phage were diluted 100-fold from stock in the experimental suspending medium to give a final titre of $1{\cdot}8$–$2{\cdot}0 \times 10^{10}$ p.f.u./ml. Much higher initial titres of experimental samples would have obviously been preferable for electron microscopy. However, initial experimental titres above 5×10^{10} p.f.u./ml. give spuriously low survivals after standard freezing and thawing procedures, probably due to phage–phage interactions as they concentrate in the unfrozen aqueous phase between the ice crystals (unpublished results). Samples of 0·1 ml. were frozen and equilibrated at −3° C. for 15 min., and then subjected to the following treatments:

(i) *Salt denaturation.* Samples suspended in 0·1 molal sodium bromide were cooled at 1° C. to predetermined subzero temperatures and then immediately thawed slowly in a polystyrene block maintained at 4° C.

(ii) *Eutectic injury.* Samples suspended in 0·1 molal phosphate buffer (eutectic temperature −4·3° C.) or 0·1 molal phosphate-buffered saline (KH_2PO_4-Na_2HPO_4-NaCl, eutectic temperature −23·5° C.) were cooled at 1° C./min. to −45° C. and thawed slowly at 4° C. A third set of samples, suspended in 0·1 molal phosphate buffer, were cooled at 1° C./min. to −23° C., stored at −23° C. for 1 hr. and finally thawed slowly at 4° C. (Phosphate buffer exerts a stabilizing effect which counteracts the denaturant effect of sodium chloride and thus eutectic injury occurs in these samples without superadded salt denaturation.)

(iii) *Mixed salt denaturation and eutectic injury.* Samples suspended in 0·1 molal sodium chloride (eutectic temperature −21·8° C.) were cooled at 1° C./min. to −23° C., and the eutectic seeded (unless otherwise indicated) by touching the frozen surface of each sample with solid eutectic precooled in liquid nitrogen vapour. (As −23° C. is just below the eutectic temperature of the suspending medium the samples remain supercooled unless seeded). Samples were stored at −23° C. for 1½ hr. and then rapidly thawed by agitation in a 37° C. waterbath.

(iv) *Mixed salt denaturation and eutectic injury with reactivation.* Seeded samples were stored at −23° C. for 1½ hr. and thawed rapidly at 37° C. as in (iii) above, and then incubated at 37° C. for 3 hr.

(v) *Mixed salt denaturation and eutectic injury with subeutectic inactivation.* Samples were cooled at 1° C./min. to −23° C., seeded with solid eutectic and then cooled at 180° C./min. to −90° C. After 15 min. storage at −90° C. the samples were either rewarmed at 2° C./min. to −25° C. and then thawed rapidly at 37° C., or thawed rapidly at 37° C. directly from −90° C.

After the treatments described in (i)–(v) standard plaque assays were carried out for each sample using quadruple plates for each determination of viability. The degree of dilution of each sample used (10^{-7} to 10^{-5}) was that which gave individual plate counts of between 50 and 500. Survivals are expressed as percentages of the control plaque counts. Specimens of each undiluted sample were also taken for electron microscopy.

Electron microscopy

Control and frozen-thawed samples were placed on carbon- and colloidin-coated copper grids (Athene 200) for 1 min. to allow for absorption, and then the absorped particles were negatively stained with 2% uranyl acetate. Bacitracin at a concentration of 25 μg./ml. was used as the wetting agent (Gregory & Pirie, 1972). The grids were examined in a Philips EM 300 electron microscope operated at 80 kV and a magnification of 42,000 and Ilford EM 4 plates were used for the production of micrographs. Differential counts of phage particles with various distinctive types of morphological changes were performed in quadruplicate, whole squares of the grid being scanned for each count. The percentage of abnormal phage particles in control counts was less than 1%.

RESULTS

Salt denaturation

The morphological changes from normal phage particles (Plate 1*a*) that were observed in phage samples which had been frozen and thawed in the sodium bromide suspending medium were contraction of the tail sheath, and variable loss of DNA which appeared as a cloud at the tip of the tail core (Plate 1*b*, *c*). There was an excellent inverse correlation between the percentage of morphologically abnormal phage particles (contracted tail sheaths) and the percentage viable phage as determined by standard plaque titre (Fig. 1).

Eutectic injury

Several distinct morphological changes were observed in phage particles from samples which had undergone a eutectic phage change, either that of phosphate buffer or of phosphate buffered saline. Rare particles were seen which showed a discontinuity in the tail sheath at 11–22 nm below the tail collar, but had an intact tail core. These particles had either full or empty heads (Plate 1*d*, *e*). Very rarely particles were observed with the sheath discontinuity whose tail core appeared to have fractured during negative staining (Plate 1*f*, *g*). These were counted as disjoined heads. The commonly observed morphological changes were complete disjunction of head and tail (Plate 1*h*, *i*, *j*). The tail core was fractured 40–60 nm. below the tail collar. Full disjoined heads were counted together since the tail

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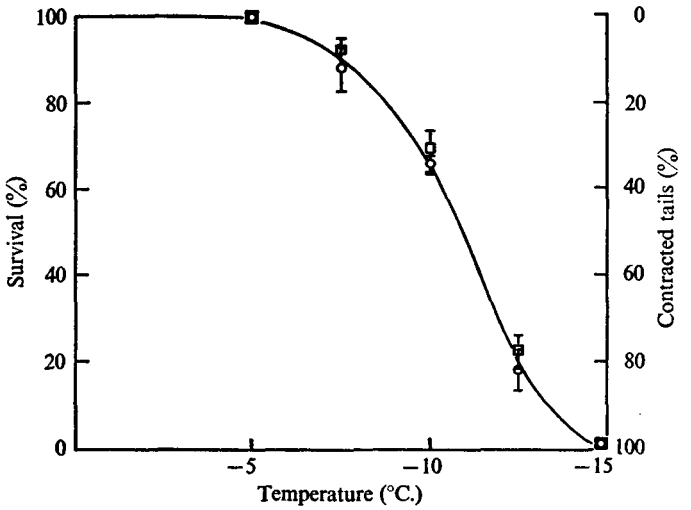

Fig. 1. T4Bo phage suspended in 0·1 molal NaBr were cooled to the indicated temperatures at 1° C./min. and then thawed slowly. The results show the percentage viabilities of the frozen–thawed samples as determined by plaque assay (○) and the percentage of particles with contracted tails counted electron-microscopically (□).

remnant was easily seen on almost all of them, whereas separate counts were made of empty heads with and without a visible tail remnant. The latter two appearances are probably of the same entity, since a collapsed head may be able to lie on the grid above the tail remnant thus obscuring it in many cases. Some difficulty was encountered in deciding into which of the latter two categories some particles should be placed. An entirely separate appearance was that of particles with normal tails, but empty heads and usually a conspicuous tear in the head membrane (Plate 1*k*). Disjoined tails (Plate 1*b*) were easily missed during a differential count and thus were not counted separately. They appeared to be as frequent in number as disjoined heads. No particles with contracted tails were seen in samples subjected to eutectic phase change injury only. Nor were any disjoined tails seen in which the sheath was in the contracted state. In all three experiments (Fig. 2A–C) the results were very similar. The percentages of morphologically normal particles corresponded fairly closely to the percentages of viable phage (plaque assay), and of the morphologically abnormal particles, the majority showed head–tail disjunction with the proportion of full:empty heads being about 1:5, while the remaining abnormal particles showed empty heads with normal tails.

Mixed salt denaturation and eutectic injury

The unseeded samples showed only contracted tails (Fig. 3A) as described above for salt denaturation. The seeded samples showed all the morphological changes described separately above under salt denaturation and eutectic phase change injury (Fig. 3B). The percentage of morphologically normal phage particles again closely corresponded to the percentage of viable phage (plaque assay). The proportion of particles with contracted tails correlated well with the number

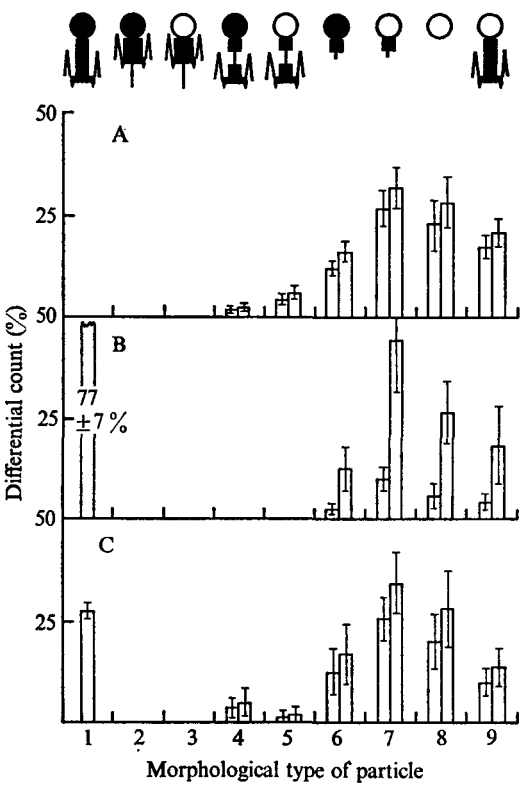

Fig. 2. Differential counts of normal and altered T4Bo phage particles following freezing and thawing. The symbols represent: 1, normal particles; 2, contracted tail, full head; 3, contracted tail, empty head; 4, disjoined tail sheath, full head; 5, disjoined tail sheath, empty head; 6, disjoined full head; 7, disjoined empty head; 8, disjoined empty head, without tail remnant; 9, empty torn head, normal tail. Vertical bars represent ± 1 standard deviation. The first value of the differential count for abnormal particles represents the percentage of the total number of particles counted, while the second value represents the percentage of the total number of abnormal particles counted. A, phosphate buffer $-45°$ C.; B, phosphate buffered saline $-45°$ C.; C, phosphate buffer $-23°$ C. The respective percentage viabilities (plaque count) were (A) $17{\cdot}2 \pm 3{\cdot}2\%$, (B) $72 \pm 6{\cdot}4\%$, (C) $18{\cdot}2 \pm 0{\cdot}2\%$. For further details see *Experimental Procedures* (ii).

observed following salt denaturation only (Fig. 3A), while the remaining abnormal particles showed in general the changes described above under eutectic injury, except that by comparison there was a conspicuously low proportion of particles with normal tails and empty torn heads. No particles were seen which had the appearance of heads disjoined from contracted tails, and no contracted disjoined tails were seen. Close examination of those particles counted as normal revealed no morphological changes from particles of normal control suspensions. The latent injury of these particles as demonstrated by hypersensitivity to repeated freezing or ultrasonic vibration could not therefore be identified morphologically. The differential count of the 'reactivated' sample (Fig. 3C) was not significantly different from that of the non-reactivated sample (Fig. 3B).

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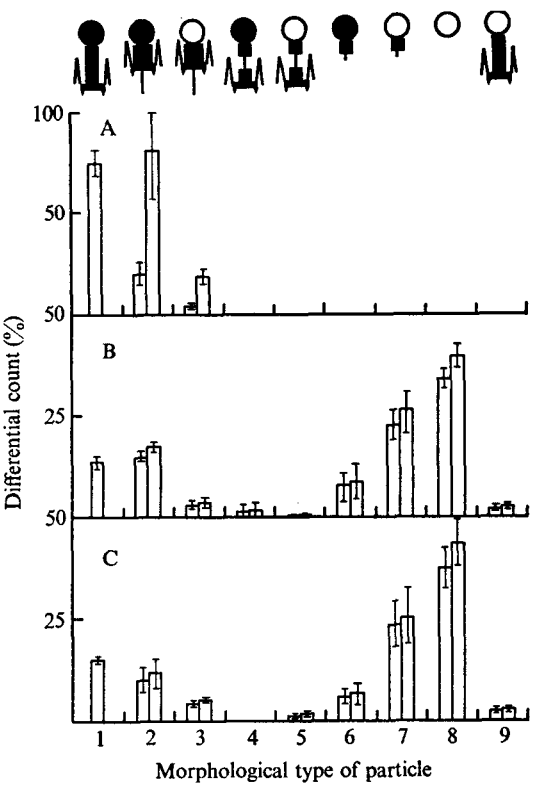

Fig. 3. Differential counts of normal and altered T4Bo phage particles following freezing and thawing. Symbols as for Fig. 3. (A) NaCl −23° C. (unseeded); (B) NaCl −23° C.; (C) NaCl −23° C. and then 37° C. for 3 hr. The respective percentage viabilities (plaque count) were (A) 76±11·7%, (B) 14·5±2·9%, (C) 12±1·4%. For further details see *Experimental Procedures* (iii) and (iv).

Mixed salt denaturation and eutectic injury with subeutectic inactivation

The samples thawed rapidly from −90° C. showed the full range of morphological changes described above for mixed salt denaturation and eutectic injury (Fig. 4A). The percentages of particles with normal tails and empty heads were higher than those observed when samples were cooled slowly to −23° C. (Fig. 3B), and much closer, to those seen following the eutectic phase changes in phosphate buffer and phosphate buffered saline (Fig. 2A–C). There was a close correlation between percentage viability and the percentage of normal particles.

In the samples thawed slowly from −90° C. no normal particles were seen electron-microscopically, and the percentage viability was < 1%. The differential count (Fig. 4B), when compared with the rapidly thawed samples, showed a shift from full to empty heads in both those particles with disjoined heads and those with contracted tails. There was little change in the proportion of particles with normal tails and empty heads. No new morphological appearances were seen.

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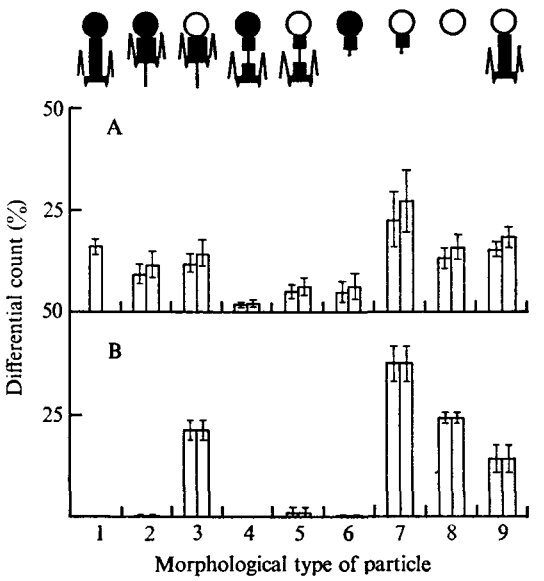

Fig. 4. Differential counts of normal and altered T4Bo phage particles following freezing and thawing. Symbols as for Fig. 3. (A) NaCl − 90° C. rapid thaw; (B) NaCl − 90° C. slow thaw. The respective percentage viabilities (plaque count) were (A) 12·1 ± 1·8%; (B) > 1%. For further details see *Experimental Procedures* (v).

DISCUSSION

Contraction of the tail sheath of T-even phage is initiated by a conformational change in the base-plate (Simon & Anderson, 1967*a*, *b*) which induces a transition of the tail sheath subunits from their extended helical symmetry to their contracted helical form (Moody, 1973). The morphological change of tail sheath contraction induced in frozen-thawed T4Bo phage by sodium bromide is well in keeping with its well-known denaturant effects. Presumably in this experimental model it brings about the conformation change in the base-plate required to trigger tail contraction.

The differential counts of the various morphologically altered types of T4Bo phage particles observed after eutectic injury only (Fig. 2A–C), suggest two separate mechanisms of injury:

(i) Subunit dissociation in the tail sheath (Plate 1*d*) followed by fracture of the tail core exposed by the sheath disjunction (Plate 1*f–j*).

(ii) Dissociation of subunits of the head membrane to produce a tear through which the DNA escapes (Plate 1*k*).

It is obvious from the differential counts that head–tail disjunction does not automatically lead to loss of DNA from the disjoined head. If head–tail disjunction and loss of DNA from heads through a tear in the head membrane do in fact occur as separate entities then the percentage of abnormal particles which show both head–tail disjunction and empty heads should be the product of the total percentage of particles which exhibit either lesion. Thus, in Fig. 2A the total percentage of abnormal particles with empty heads is 84·5% and the corresponding

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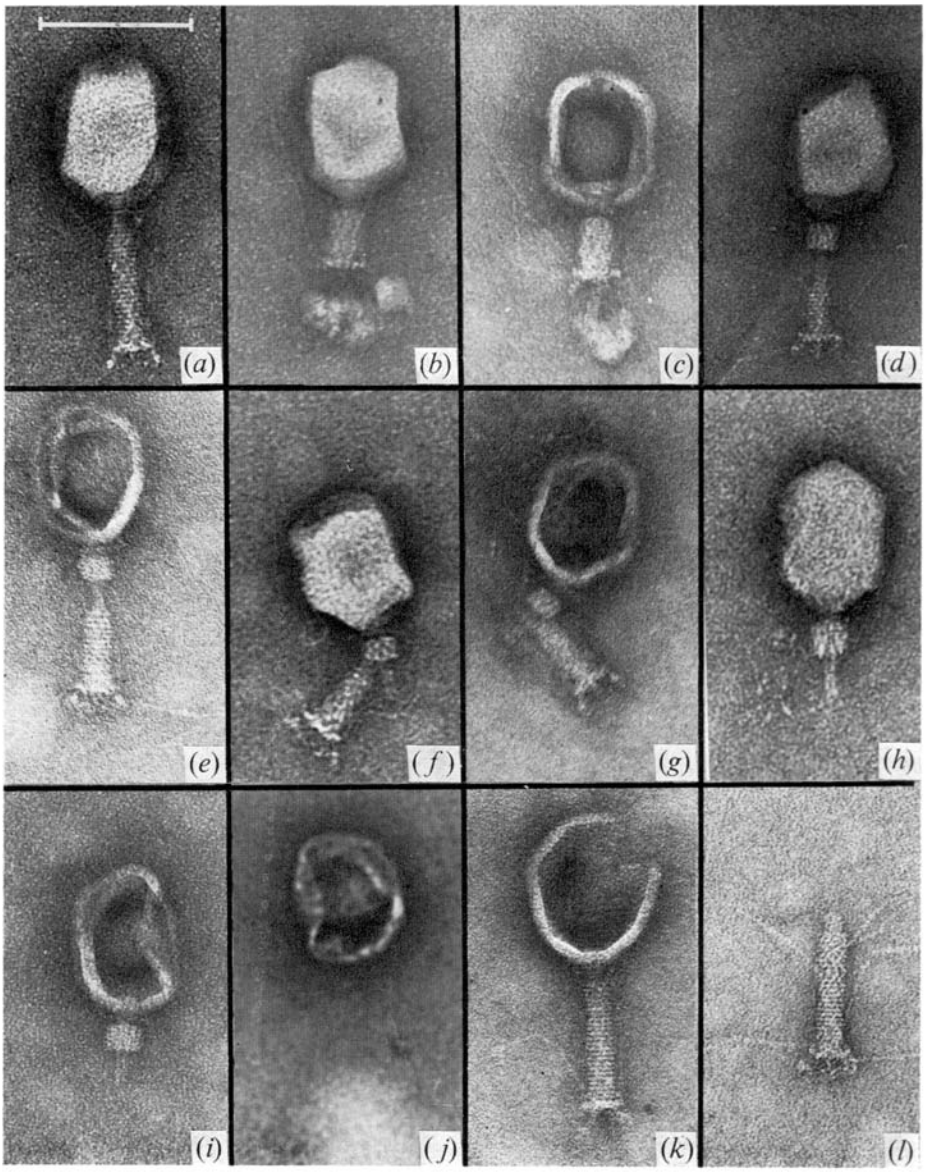
figure for particles with head–tail disjunction is 80%. If these lesions occur independently the percentage of particles exhibiting both lesions would be expected to be 68%. The actual percentage was 64·5%. The expected and actual figure (in parentheses) worked out in this way for the data in Fig. 2B and 2C are 71·5% (69%) and 67·5% (64·5%). These figures strongly indicate that head–tail disjunction and loss of DNA caused by eutectic injury are independent lesions.

Any hypothesis which proposes to provide an explanation for the way in which a eutectic phase change can result in head membrane tears and tail sheath breaks followed by head–tail disjunction (tail core break) must account for the lack of multiple lesions in individual particles (e.g. several breaks in tail sheaths or several tears in the head membrane) since these were never observed in the present experiments. The eutectic phase change inevitably causes dehydration of the phage particles, since following the phase change they are supported in a solid phase of salt and ice crystals, with the ice crystals acting as a condenser. It is proposed that this dehydration brings about increased tension between adjacent structural subunits resulting in the observed tail sheath disjunctions and head membrane tears. Once these lesions have occurred the tensions between the subunits are relaxed and thus multiple lesions are not seen in individual phage particles.

The helical symmetry of the tail sheath is believed to be identical to that of the tail core (Moody, 1973), and it is therefore a possibility that the break of the tail core following tail sheath disjunction is potentiated by mechanical weakness following loss of stabilizing core-sheath interactions between the coincident helices.

Tails which were disjoined from phage heads as a result of eutectic injury were always observed to have the sheath in the extended state (Plate 1*l*). This supports the belief that tail sheath contraction will only take place following a triggering conformational change in the base-plate.

The phage samples which were inactivated by combined salt denaturation and eutectic injury (Figs. 3A–C, 4A) showed an almost constant proportion of particles with contracted tails representing phage inactivated by salt denaturation above the eutectic temperature. It can only be assumed that contraction of the tail sheath protects both the sheath and core from disjunction caused by the eutectic phase change. It is very obvious that the ratio of empty to full heads in particles with contracted tails was much lower than that for the other abnormal particles. The particles scored as having a full head and contracted tail, did in fact always have a small cloud of DNA at their core tips (Plate 1*b*) and thus although their heads were electron-dense they contained less than their full quota of DNA. This may provide the explanation for the low empty/full head ratio noted above, in that the proposed dehydration-induced tension in the head membrane would be less in heads lacking their full quota of DNA. The differential counts of the other abnormal particles show a lower percentage of abnormal particles with empty heads in the data of Fig. 3B, C than was observed in Fig. 2A–C or Fig. 4A, B. This indicates that there was more extensive tail disjunction in those samples which were suspended in sodium chloride and stored at $-23°$ C. after seeding the eutectic. Perhaps this more extensive tail disjunction is a result of the relatively longer time taken for all the eutectic to solidify in these samples stored just below



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the eutectic temperature (−21·8° C.) than in the other samples which were cooled well below their eutectic temperatures to −45 or −90° C.

The T4Bo phage which were suspended in sodium chloride and cooled rapidly to −90° C. from −23° C. and thawed slowly showed a large increase in abnormal particles with empty heads when compared with samples thawed rapidly from −90° C., indicating that the slow thawing caused a larger proportion of the particles to have head membrane tears than rapid thawing. Indeed in this experiment virtually all the phage particles with contracted tails resulting from salt denaturation above the eutectic temperature lost their DNA when thawed slowly from −90° C. (Fig. 4B), in contrast to their anomalous retention of DNA noted earlier. There were no morphological appearances in the slowly thawed particles which had not been seen in samples exposed to combined salt denaturation and eutectic injury, indicating that the mechanism of this rapid freeze–slow thaw inactivation below the eutectic temperature is not fundamentally a separate entity.

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EXPLANATION OF PLATE

Electron microscopic appearances of frozen-thawed T4Bo phage particles. The white bar in (*a*) represents 100 nm. (*b*)–(*l*) are at the same magnification. (*a*) Normal virus particle; (*b*) contracted tail, full head; (*c*) contracted tail, empty head; (*d*) disjoined tail sheath, full head; (*e*) disjoined tail sheath, empty head; (*f*) fractured tail core, full head; (*g*) fractured tail core, empty head; (*h*) disjoined full head; (*i*) disjoined empty head; (*j*) disjoined empty head, without tail remnant; (*k*) empty torn head, normal tail; (*l*) disjoined tail.