AN UNSTABLE NUCLEAR CONDITION IN TETRAHYMENA PYRIFORMIS[1]

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CONJUGATION among the cells of a single clone has been observed in many Ciliates, including those in which mating types have been demonstrated. In forms for which mating types have not been established, two interpretations of intra-clonal conjugation are possible: either the cells that unite in conjugation are physiologically identical, or they are physiologically distinct (of diverse mating types) but the physiological distinctions are transitory. In forms with mating types, intra-clonal conjugation has usually been shown to involve the union of cells of diverse physiological types (KIMBALL 1939; JENNINGS 1941) which may be perpetuated with some degree of reliability in vegetative growth. Since mating types are involved in the "selfing" of some Ciliates, it has been proposed (KIMBALL 1943) that selfing in other Ciliates has a similar basis and that all, or nearly all, Ciliates possess mating types. This is, however, only the simplest interpretation and must be accepted with reservation.

Whatever may be the explanation for selfing in the majority of Ciliates, the presence of diverse cell types in clonal cultures of Paramecium (KIMBALL 1939) and Tetrahymena (NANNEY and CAUGHEY 1953) provides an opportunity for the study of cellular differentiation and cellular heredity. More specifically, selfing cultures present a special problem in an understanding of mating type determination and inheritance in these forms. Recently (NANNEY 1953a) a formal explanation for the selfers in *P. aurelia* was presented. The present communication, based on studies of selfers in Tetrahymena, suggests that the explanation proposed for *P. aurelia* is not applicable to Tetrahymena and that it may not be applicable to Paramecium.

MATERIALS AND METHODS

Strains of the taxonomic species *Tetrahymena pyriformis* may be divided into three general groups: 1) those in which conjugation has never been observed, 2) those in which conjugation usually occurs only when clones are mixed in certain combinations and 3) those in which conjugation occurs regularly in clonal cultures. The present report is based on a series of strains belonging to the second group and isolated from a source near Woods Hole, Massachusetts, by ELLIOTT and GRUCHY (1952). From two of these original strains (WH-6 and WH-14) 7 different pure mating types have been derived (NANNEY and CAUGHEY 1953). These types were designated by Roman numerals I–VII. ELLIOTT and GRUCHY designated the first types as I and II and these are homologous with types I and II in the system of 7 types, but ELLIOTT and

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HAYES (1953) have subsequently reported a third mating type, mating type III, isolated from the same locality. This type III has been found to be homologous with type VI in the NANNEY-CAUGHEY system. In order to avoid further confusion in the literature, the designations III and VI in the NANNEY-CAUGHEY system have been exchanged. Therefore, the mating type designations in this and subsequent papers should be equivalent to those used by ELLIOTT and his co-workers.

Although these forms will grow on a defined medium (ELLIOTT and HAYES 1953), most of the work reported here was carried out on cultures grown in Cerophyl infusions inoculated with *Aerobacter aerogenes*. In a few of the experiments Cerophyl was replaced with baked lettuce or *Aerobacter aerogenes* was replaced with *Bacillus cadaveris*. The general culture methods follow closely those described by SONNEBORN (1950) for *Paramecium aurelia*.

The cytological events at conjugation in these strains (ELLIOTT and HAYES 1953; NANNEY and CAUGHEY 1953) resemble closely those described for *Leucophrys patula* (MAUPAS 1889) and for the AA strains of *Tetrahymena* (NANNEY 1953b). Cytological studies of clones under various conditions have failed to provide evidence for autogamy. An immature period occurs immediately following conjugation and is terminated by about 80 fissions. The procedure used to obtain mature exconjugant clones is to allow the exconjugants to pass through a series of isolation cultures, single cells being transferred to fresh medium in depression slides every day or two. After 24 hours at 26°C the cells have usually undergone about 10 fissions and are still actively dividing; after 48 hours they have undergone about 12 fissions and are moderately starved. The cultures from which isolations have been made ("left-over" cultures) are retained for several days and are observed periodically for selfing. Under these conditions the clones become mature after about 8 transfers and are then tubed.

Subsequently samples of each tube culture are mixed with samples of each of the seven standard mating types in order to determine the mating type of the clone. Each mixture consists of about 0.25 ml of the well-fed unknown culture and an equal quantity of a well-fed standard type. The controls consist of the unmixed unknown culture, the unmixed standards and mixtures of the standards in all 21 combinations of 2. Since mating does not occur immediately, the tests are examined for conjugants at several times on the following day. Pairs usually form over a period of several hours and may remain together as long as 18 hours; hence, it appears unlikely that pairs occurring in a mixture will fail to be observed. An unknown culture is classified as of a particular type if it fails to mate with the standard of that type, but mates with all the other mating types. A culture is classified as a selfer if pairs are formed in the unmixed control; such cultures also show pairs in all the mixtures with the standards. Many selfers are detected before mating type tests are set by observing the left-over cultures in the isolation series. A small fraction of the cultures may remain undetermined on the first test, i.e., they may fail to mate with two or more of the standards. When such cultures are fed in the test depressions with inoculated culture medium, their mating types may usually be assigned on the following day.

OBSERVATIONS

The nature of selfing clones

Nearly all the crosses thus far made have yielded some selfing clones. The first question raised concerns the reason for the selfing: are the cells that conjugate in clonal cultures of the same or of different mating types? Evidence has been presented previously (NANNEY and CAUGHEY 1953) to show that when cells of diverse mating types are mixed, conjugation occurs only between cells of unlike mating type. In light of this evidence the simplest explanation for conjugation in clonal cultures is that these clones contain cells of two or more mating types. This explanation is supported by the fact that stable cultures of several different mating types have been derived from some of the selfing clones. Single cells isolated from selfing cultures yield some sub-cultures which again self, and some sub-cultures which are pure for some mating type and remain so. (Still other isolations yield immature clones; such isolations are obtained only from cultures in which conjugation has been permitted and are presumably due to the existence of exconjugant cells in the cultures. Such isolations are excluded in the following discussion.) The 5 pure sub-cultures from one selfing clone included 4 different mating types—II, IV, V and VI. The 9 pure sub-cultures from another clone also included 4 types—III, IV, VI and VII. Sub-cultures from other selfing clones yielded 2 or 3 different mating types in various combinations and with various relative frequencies. In these cases it is clear that a diversity of types occurs within the cultures and that this diversity is sufficient to account for the observed conjugation.

These observations do not, however, rule out the possibility that some of the conjugation observed in selfing clones is due to the union of cells of the same mating type. This possibility would not require serious consideration were it not for the fact that it has not been possible to derive diverse pure types from some of the selfers. In several instances where 15 to 30 pure sub-cultures were derived from a selfer, all were of the same mating type. The selfer most intensively studied produced over 200 pure sub-cultures, all of mating type VI. It is possible that cells conjugating in these clones have the same mating type, but this conclusion is not required. The failure to establish pure cultures of more than one mating type from a selfer may be due to the rarity or the instability of one or more of the types. If a particular type were rare, it would be selectively involved in conjugation so that among the cells which were not conjugating at the time isolations were made, it would be virtually absent. If a particular type were unstable, an isolated cell manifesting that type might again give rise to a selfing culture or even a pure culture of a different type. Since diverse mating types have been shown to occur in some selfing cultures and since no convincing evidence is available for conjugation involving cells of the same mating type, it will be assumed tentatively in the following discussion that two or more mating types occur in all selfing cultures.

The origin of mating type diversities in selfing clones

The second question raised in regard to the selfers concerns the manner in which the diversities arise. Mating types are known to change at autogamy in *P. aurelia*

(KIMBALL 1937; SONNEBORN 1947). Since the mode of mating type determination in Tetrahymena resembles so closely that in *P. aurelia*, it would be expected that autogamy, if it occurred in *T. pyriformis*, would result in the formation of diverse mating types. Therefore, attempts have been made to detect autogamy in the WH strains and particularly in the selfing clones. Several clones in which selfing had been observed in left-over cultures were chosen for study. Single cell isolations from well-fed selfers usually give rise to cultures which self extensively when allowed to starve. If autogamy is to account for this selfing, it must occur between the time a single cell is isolated and the time the culture starves some 12 fissions later. Large numbers of cells were removed from such cultures both while they were growing rapidly and after they had begun to starve. No evidence was found for reorganization in unpaired cells, yet extensive selfing, involving more than half the cells, occurred in all the cultures studied. Diverse pure types were subsequently obtained from some of these same cultures. While it remains possible that autogamy occurs rarely in these strains, it must be concluded that autogamy is not a major factor in rendering cells in selfing cultures capable of conjugating.

Finally it is possible that conjugation (or some other process of nuclear reorganization occurring at the time of conjugation) is the source, rather than the result of mating type diversities. This is considered unlikely in view of the fact that conjugation normally results in cells which are immature for long periods of time; the diversities now known to be produced at conjugation cannot account for the diverse mature types derived directly from selfing clones. It was considered necessary, however, to obtain diverse pure cultures from selfers in which conjugation had not yet been permitted. This was accomplished as follows. Clones were maintained in daily transfers until selfing was observed in left-over cultures. When selfing was observed, single cells were isolated from the next culture in the isolation series, a culture in which the cells were well-fed and in which conjugation had not yet occurred. Under such circumstances relatively few cells give rise to pure cultures, but cultures of diverse pure type were obtained from some of the selfers. This shows clearly that neither conjugation nor some other process of reorganization associated with conjugation is solely responsible for the mating type diversities observed in selfing clones. The diversities appear during cytologically normal vegetative growth.

The stabilization of selfing clones

The experiments mentioned in the previous paragraph also demonstrated another fact—that cells isolated from selfers under different conditions yield different frequencies of pure cultures. 208 cells isolated from 7 different well-fed selfers produced only 14 (7%) pure cultures. 204 cultures derived from these same clones when starved included 58 (28%) pure cultures. Every selfer examined produced a higher frequency of pure cultures from starved cells than from well-fed cells.

At least two explanations for this observation seem possible. Either starvation (or some condition associated with starvation) is responsible for stabilizing the mating types of cells in selfing clones, or those cells more likely to give rise to selfing cultures are also more likely to participate in conjugation and are, hence, selected against in making isolations from cultures in which conjugation is occurring or has occurred,

i.e., starved cultures. This latter explanation is rendered improbable by the fact that cells can be stabilized by starvation under conditions which permit no conjugation and, hence, no obvious selection. 312 cells from a total of 15 well-fed selfers were isolated into culture medium and allowed to grow; only 21 (7%) of the derived cultures were pure for a mating type. 416 cells from these same cultures were individually isolated at the same time in separate drops of distilled water and allowed to starve; these cells, after being isolated into culture medium, produced 119 (29%) pure cultures. Such observations strongly indicate that starvation may stabilize potential selfers and that the starvation effect is not due to crowded culture conditions or accumulated metabolites.

Since starvation was shown to have a marked effect on selfers after the onset of selfing, it was considered of interest to determine whether a starvation effect could also be demonstrated at a time before overt selfing had occurred. This possibility was first examined by differential treatment of the progeny of unselected crosses. In one series of 15 crosses, representing 11 different parental mating type combinations, the exconjugant clones were transferred daily for ten days as single cells to new media and were then tubed and tested for mating type. During the growth period the clones were not allowed to starve, except in the left-over cultures. The left-over cultures were examined for conjugants every 24 hours during the interval in which they were starving. Of the 360 clones studied, 69 (19%) were observed selfing after they became mature.

The progeny of another series of 28 crosses, representing 14 combinations of parental mating types, were treated in precisely the same fashion as the previous series with the exception that single cells were transferred on alternate days, after the isolation cultures had begun to starve. Only 16 (2%) of the 758 clones were selfers at the time of maturity. These experiments strongly indicated that starvation during growth had an effect on the frequency of selfing detected after maturity. Certain criticisms can, however, be raised against such data, and certain questions remain unanswered. The chief criticisms are concerned with the fact that the crosses used in the two series were not precisely comparable and that the experimental clones were studied at different times over a period of several months. Moreover, the numbers of progeny in the individual crosses were so small as to make difficult any test of homogeneity within the two series. An experiment was designed, therefore, specifically to answer the question of whether starvation prior to selfing had an influence on the incidence of selfing.

For this experiment a cross was made between sister caryonides of a highly inbred strain; the exconjugants and the first fission products were separated to provide 4 caryonides from each original pair. After the caryonides had undergone about 10 fissions two cells were isolated from each caryonidal culture to give rise to two sub-cultures designated as A and B. The A sub-cultures were subsequently transferred daily to new depressions until they had undergone a total of 60 fissions. The B sub-cultures were transferred at 3-day intervals until they had also undergone a total of 60 fissions. After 60 fissions both the A and B sub-cultures were again split into two further sub-cultures, A-1 and A-2, B-1 and B-2. The A-1 and B-1 cultures were maintained thereafter in daily isolations until they had undergone a total of 160 fissions

from conjugation. The A-2 and B-2 cultures were transferred at 3-day intervals until they also had undergone a total of 160 fissions. All 4 series were examined at 12 hour intervals as the left-over cultures starved in order to detect the occurrence of selfing. The A-1 series, which was not allowed to starve at any time during the 160 fissions, produced 77 (56%) selfers among 138 surviving clones. The A-2 series, which was permitted to starve only after the first 60 fissions, produced 21 (16%) selfers among 134 clones. The B-1 series, starved only before the 60th fission, produced only 5 (4%) selfers among 136 clones, and the B-2 series, starved at every transfer, produced 9 (7%) selfers among 131 clones. These results leave little doubt that starvation prior to the onset of selfing decreases the observed frequency of selfing.

The relationship between maturity and instability

This experiment also yields information regarding the time when starvation can be effective in relation to the end of the period of immaturity. The cross used was one which could not yield mating type I; in order to detect the onset of maturity, the left-over cultures in the A-1 and B-1 series were mixed with well-fed mating type I cultures. After 40 fissions in the A series practically none of the clones was reactive; after 50 fissions a few (16%) were beginning to be reactive; after 60 fissions 43% were reactive to some extent and after 70 fissions 83% were reactive; after 80 fissions all the clones were completely reactive. In terms of fissions, maturity arrived somewhat earlier in the B series; all the B clones were completely reactive by 60 fissions, the first time they were tested. The rationale for using the 60th fission as a point of bifurcation in the experiment was to determine whether starvation was effective in preventing selfing if applied either before or after the onset of maturity. Since maturity does not arrive abruptly, when considering either the total series or individual clones, 60 fissions is only an approximation of the transition from immaturity to maturity. Nevertheless, the data strongly suggest that starvation either before or after the beginning of maturity is effective in stabilizing potential selfers.

This experiment also shows one additional characteristic of the selfers, the relationship between the onset of maturity and the onset of selfing. Again referring to the A-1 series, selfing was never observed as soon as the culture became mature. Some clones (about 10% of the detected selfers) conjugated in the isolation culture immediately following the one for which maturity was first demonstrated. These clones may have included diverse mating types when they became mature and the failure to detect pairs earlier may have been due to their relative immaturity in the previous isolation culture. This conclusion appears much less likely in the case of clones which did not self for many fissions after they became mature. Three clones selfed for the first time 90 fissions after they became mature and the average number of fissions between the onset of maturity and the detection of selfing was about 40 fissions. It is conceivable that some of this apparent lag between maturity and selfing was due to faulty observation, but since the left-over cultures were examined carefully at 12 hour intervals it seems improbable that any large amount of the selfing was missed.

DISCUSSION

The physical basis for selfing in the Ciliates

A previous attempt (NANNEY 1953a) to provide a formal explanation for the occurrence and behavior of selfers in *P. aurelia* was based on the following considerations. 1) Mating types in this species are controlled by the macronuclei. The studies of SONNEBORN (1947, 1951) have provided abundant evidence for this conclusion, the chief evidence being that in certain stocks new mating types arise only when new macronuclei are formed and the separation of diverse mating types occurs only when the new macronuclei are segregated, i.e., at the first fission following nuclear reorganization when caryonides are separated. 2) The exceptions (rare in some stocks and varieties and common in other stocks and varieties) were considered exceptions only in a qualified sense. Selfing, like mating types, was thought to be a caryonidal characteristic and, hence, related to some macronuclear peculiarity. Critical evidence is not available to show that selfing in *P. aurelia* is caryonidal, but the fact that mating types are controlled by the macronuclei suggests that the aberrant mating type control in selfing clones is related to some macronuclear peculiarity. 3) A macronucleus undoubtedly contains many sets of nuclear genes (SONNEBORN 1947). The manner in which these are organized in the macronucleus is not known, but it is possible that they form sub-structures with some degree of integrity. 4) If the unit of mating type determination were the sub-nuclei rather than the macronucleus as a whole, two classes of macronuclei would be conceivable: those that consist of a single type of sub-nucleus, determine a single mating type and reproduce true to type and those that contain more than one type of sub-nucleus, which have the potentialities for more than one mating type and which can produce, through the segregation of sub-nuclear units at fission, cells of diverse mating types. This interpretation held that the "instability" of selfing clones was due to structural inhomogeneity of the macronucleus and that the ultimate unit of mating type determination, the sub-nucleus, was not unstable after it had been determined, i.e., after the macronucleus had been formed. This general explanation for selfing in *P. aurelia* appeared to satisfy most of the observations concerning selfers in this species.

Two further lines of evidence (NANNEY 1953a) were set forth as additional support for the hypothesis of structural inhomogeneity. It was argued that if spontaneously arising unstable macronuclei were composed of smaller macronuclear elements of diverse kinds, then selfers should be induced by the fusion of macronuclei which would ordinarily control different mating types. Selfers were induced by this method and appeared to show all the characteristics of spontaneous selfers. Secondly it was argued that small pieces of a "selfing" macronucleus would be more likely to be homogeneous than large pieces of the same macronucleus; hence, if a macronucleus were allowed to break up into small pieces and if these small pieces were allowed to regenerate (see SONNEBORN 1947, 1950), such regenerated fragments would be more likely to control a pure mating type than would the much larger pieces separated at normal fission. This expectation was experimentally verified (SONNEBORN unpublished; NANNEY unpublished).

Preliminary data (NANNEY and CAUGHEY 1953, and unpublished) indicate that the mechanism of mating type determination in *T. pyriformis* is very similar to that in *P. aurelia* (specifically, the Group A varieties of *P. aurelia*). Some of the characteristics of these similar systems of mating type determination are as follows. 1) The mating types are not determined solely by the genotypes of the cells. 2) The unit of mating type inheritance is the caryonide. 3) The temperature prevailing at the time the new macronuclei are developing has some influence on the probability of origin of a macronucleus controlling a particular mating type. 4) Unstable clones may occur and from such unstable clones diverse mating types may be extracted.

Since mating types in both these forms show caryonidal inheritance, it appears highly probable that nuclear differentiation of a similar nature is involved in the determination of mating types in the two forms. Therefore, any insight into the nature of the macronuclear differences in one form should be pertinent to a discussion of the differences in the other form. In this connection the recently proposed gene-dosage hypothesis for mating type differences in *P. aurelia* (NANNEY 1953a) requires re-examination. The simplest application of this hypothesis held that one mating type was characterized by twice as much genetic material as the other. This interpretation is at least conceivable in a form, such as *P. aurelia*, in which only two mating types exist per variety, but becomes extremely improbable in a form with as many as seven different types per variety. This consideration aside, the gene-dosage hypothesis in any simple form now appears untenable. SONNEBORN (1953) has shown that certain breeding results expected on the basis of the hypothesis are not found. Moreover, it has not been possible to demonstrate any consistent difference in the desoxyribose nucleic acid content of cells of different mating type in *P. aurelia* (GUTHE, TEFANKJIAN and NANNEY unpublished). For these reasons it seems advisable to abandon the gene-dosage hypothesis for *P. aurelia* and to consider as essentially similar the systems of mating type determination in *P. aurelia* and *T. pyriformis*.

The hypothesis of structural inhomogeneity (which was independent of the gene-dosage hypothesis) appeared to offer an opportunity for the analysis of the pattern of segregation in selfing clones. The probability of origin in such clones of a macronucleus containing only one type of sub-nucleus, and hence controlling a single pure mating type, would depend upon the total number of sub-nuclei present, the original proportions of the sub-nuclear types and the manner in which the sub-nuclei were separated at nuclear division. An examination of the segregation pattern was, however, difficult in *P. aurelia* because of a relatively short period of vegetative growth before autogamy intervenes and replaces the old macronucleus. The occurrence of selfing and the apparent absence of autogamy in Tetrahymena suggested that here a detailed analysis of the segregation pattern would be feasible. In attempting to study this pattern of segregation it was observed that starvation of selfing clones results in their becoming stable. These results were not expected on the hypothesis of structural inhomogeneity. A macronucleus composed of stable sub-nuclei of diverse kinds could not be transformed into one with only one sub-nuclear type by starvation unless a severe and undemonstrated intra-nuclear selection were involved. The idea that the sub-nuclei are the units of mating type determination

loses its utility, since the sub-nuclei, like the macronucleus as a whole, would have to remain plastic.

The selfers in *P. aurelia* appear to be essentially like those in Tetrahymena. Recent studies (RUDNYANSKY unpublished) suggest that stabilization of selfing clones in this species may also be accomplished by certain treatments. It is possible, however, that more than a single physical basis for selfing exists, that selfing in *P. aurelia* is due to structural inhomogeneity and selfing in Tetrahymena is due to physiological instability. The simplest interpretation of present data would hold, however, that selfing in both species is due to physiological instability of the macronuclei. A similar proposal has been put forth (BUTZEL 1953) independently and on other grounds for the mating types in *P. aurelia.*

Progressive nuclear differentiation in Tetrahymena

The fact of caryonidal inheritance of mating types in Tetrahymena (NANNEY and CAUGHEY 1953, and unpublished) establishes the macronucleus as the focus of mating type determination in this form. Since cells derived from a single exconjugant may show several different mating types, it is clear that cells with identical genotypes may manifest any of several different phenotypes. Evidence now available (NANNEY and CAUGHEY unpublished) demonstrates that one or more genetic loci are involved in determining the mating type potentialities. A central problem is, therefore, the manner in which one of the various gene-controlled potentialities is established to the exclusion of the others. Although present data are not adequate to provide a completely satisfactory answer to this problem, certain characteristics of the nuclear differentiation may be discussed.

The nuclei from which the new macronuclei are derived undoubtedly possess the full spectrum of potentialities. The time at which an exclusion of potentialities is begun is not known with certainty, but it may be correlated with the time of morphological differentiation, when the presumptive macronuclei begin to enlarge. This morphological nuclear differentiation is certainly under the control of the cytoplasm surrounding the nuclei at this time (NANNEY 1953b), but no good evidence is available to show that the cytoplasm has any specific effect in directing the differentiation of the macronucleus in regard to mating type. In this respect the studied strains of *T. pyriformis* resemble the Group A rather than the Group B varieties of *P. aurelia* (SONNEBORN 1947; NANNEY 1954). During this primary stage of development it is possible to direct to some extent the course of nuclear differentiation by means of temperature (NANNEY and CAUGHEY 1953); the frequencies of the types observed at maturity depends upon the temperature at which conjugation and/or macronuclear development occurs.

The two new macronuclei in an exconjugant are separated at the first post-zygotic cell division. That the nuclei are at least partially differentiated by this time is shown by the fact that the various sub-lines (sub-caryonides) derived from one of these first fission products are strongly correlated in their mating types while the sub-lines derived from diverse caryonides are not correlated. That the macronuclei are not completely differentiated at this time is shown by the fact that several different mating types *may* be derived from a single first fission product. Thus, by the time of the first

post-zygotic cell division the nuclei have passed from an originally totipotent state to one in which the mating type potentialities are severely restricted.

At the time of maturity, many fissions later, over half the nuclei may still possess multiple potentialities but one potentiality is usually predominant. A final exclusion of potentialities may be brought about by the starvation of the cells. The mechanism of this exclusion is not known, but once exclusion is complete the nuclei only rarely, if ever, return to a pluripotent condition. Present data suggest that this final limitation of potentialities is not related to those processes responsible for the maturation of the cells.

SUMMARY

1. Intra-clonal conjugation (selfing) in the WH strains of *Tetrahymena pyriformis* is shown to be due, in many cases at least, to the presence of two or more mating types within a clonal culture.

2. The mating type diversities which are detected in clonal cultures arise during cytologically normal vegetative growth.

3. The cells in such unstable clones may be stabilized, i.e., may lose the capacity to produce several mating types, if they are permitted to starve.

4. This stabilization by starvation can apparently occur over a wide range of time during the growth of the clones, whether or not the cells are mature and whether or not selfing has been detected in the cultures.

5. These observations, in conjunction with others previously reported, are interpreted as indicating a progressive nuclear differentiation whereby the potentialities of a clone are gradually delimited. Certain aspects of this differentiation are under experimental control.

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