GENETICAL AND BIOCHEMICAL STUDIES ON DEVELOPMENT OF THE CELLULAR SLIME MOLD, *Dictyostelium discoideum*.

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CONTENTS

PART I

ISOLATION AND CHARACTERIZATION OF AGGREGATELESS MUTANT, HT41.

INTRODUCTION

In the cellular slime mould Dictyostelium discoideum, one of the first important steps in its development is the process of cell aggregation triggered by starvation. During several hours of starvation amoebae gradually acquire aggregation competence. They first become sensitive to the aggregation signal, cyclic AMP (cAMP) (Konijin et al., 1968; Robertson et al., 1972). This is probably due to either a greater number of, or more sensitive cAMP receptors on the cell surface, and increase of a cAMP-destroying enzyme, phosphodiesterase (Malchow et al., 1972; Riedel et al., 1973). A few hours later cells acquire the ability to relay the signal, that is, they synthesize and release cAMP when stimulated by exogenous cAMP (Robertson et al., 1972; Roos et al., 1975). Subsequently some cells appear in the cell population which emit cAMP autonomously and rhythmically (Robertson et al., 1972; Gerisch & Wick, 1975). As periodically emitted cAMP acts not only as a signal for cell aggregation but also as a stimulus for cell differentiation, cells surrounding the signaling-cells gain enhanced chemotactic responce, and move actively toward the source of stimuli. They also acquire new cohesiveness which is necessary for cell contacts (Gerisch et al., 1975; Darmon et al., 1975). These steps lead to collection of a large number of cells toward the central points and

construction of multicellular aggregates.

At present, several questions remain unanswered concerning the process of cell aggregation, such as the appearance of signaling-cells, and the mechanisms of autonomous signaling. Analysis of mutants which are defective in either signal relaying or autonomous signaling may elucidate some important mechanisms in aggregation. We have isolated a mutant which has defects in both functions. In this paper we describe and discuss the characteristics of this mutant. It is a useful strain for understanding the basal mechanisms underlying cell aggregation.

MATERIALS and METHODS

Strains and culture conditions. Experiments were performed with Dictyostelium discoideum strain NP14, which was kindly supplied by Dr. P. C. Newell, Oxford University, and aggregateless mutants isolated from NP14 in this laboratory. NP14 cells aggregate and construct normally white fruiting bodies. This strain was used as a standard strain throughout the experiments.

Cells were grown with Aerobacter aerogenes on nutrient agar at 22 C (Hashimoto et al., 1976). Growing amoebae were harvested using either LPS buffer (Sussman, 1966) or 17 mM phosphate buffer, pH 6.0, washed three times by centrifugation and resuspended in the same buffer. Number of cells was counted using a haemocytometer.

Mutagenesis. Freshly constructed fruiting bodies were irradiated with ^{60}Co γ-ray at 3.7-5.0 x 10^5 R and developmentally deficient mutants were isolated (Yamada et al., 1973).

Time-lapse cinematography. Washed amoebae were placed on non-nutrient agar plates at a low density of 2 x 10^5 cells cm^{-2} to observe the movement of the individual cells. Films were taken at a constant temperature with a Bolex 16 mm movie camera and a Nikon CFMA camera drive. Kodak

4-X 16 mm reversal films were used for recording. Frame rate was 2 frames min^{-1}, and film magnifications between 10 and 20 were used.

Light-scattering. Light-scattering changes of cell suspension were measured as described Gerisch & Hess (1974). Washed amoebae were shaken in 17 mM phosphate buffer at a density of 1 x 10^7 cells ml^{-1}. After various times of incubation, they were centrifuged and adjusted in cold buffer to 3 x 10^7 cells ml^{-1}, then transferred into a cuvette. The cell suspension was agitated by bubbling water-saturated air and recorded for light-scattering at 405 nm. Sometimes, small drops of cAMP were injected through a needle and response of cells was recorded. All measurements were done at 22 C.

Cyclic AMP pulses on a cell layer. Amoebae were harvested, washed and plated on non-nutrient agar at a density of 1 x 10^7 cells cm^{-2}. After they adhered to the surface, 5 ul of cAMP solution (10^{-4} M) was added dropwise on a cell layer at time intervals of 5 min with the use of an electrically controlled peristaltic pump. After 15 hr the agar plates were examined under a microscope.

Intercellular interaction between NP14 and aggregate-

less strain. Capacity of the aggregateless mutant to cosporulate with NP14 was examined. NP14 cells were mixed with aggregateless mutant cells at a ratio of 1 : 5. The mixture was plated on non-nutrient agar plates at 1 x 10^7 cells cm^{-2} and incubated at 22 C. After 48 hr spores were collected from the fruiting bodies and plated clonally on nutrient agar with the bacteria after appropriate dilutions. Plaques were examined in their ability to aggregate.

Extracellular cAMP-phosphodiesterase activity. Extracellular fractions were collected according to a modified method of Riedel et al. (1973). The growing amoebae were freed from the bacteria, suspended in LPS buffer at 1 x 10^7 cells ml^{-1} and shaken at 22 C. After aliquots were removed at various times during development and centrifuged at 3000 rev. min^{-1} for 30 min, the supernatants were dialyzed overnight against 10^{-2} M Tris-HCl buffer, pH 7.5. Enzyme activity was assayed according to the methods described by Yanagisawa et al. (1974).

Assay for contact sites A. Cell contact which is resistant to EDTA was assayed according to the methods of Beug et al. (1973). Washed cells were shaken in 17 mM phosphate buffer at 1 x 10^7 cells ml^{-1}. Aliquots were taken at various times and EDTA was added at final

concentration of 10^{-2} M. After shaking to dissociate possible agglutinates, the number of total cells and dissociated cells was counted with a haemocytometer. The proportion of single and double cells was calculated.

Effects of cAMP pulses on the accumulation of extracellular phosphodiesterase and EDTA-resistant cell contacts. Mutant cells were washed, suspended and shaken in phosphate buffer at 1×10^7 cells ml^{-1}. Small drops of cAMP solution were added at time intervals of 5 min (Darmon et al., 1975). Final concentration of cAMP in the suspension, after addition of one drop, was 1×10^{-7} M. At appropriate times, activity of extracellular phosphodiesterase and EDTA-resistant cell contacts were assayed.

RESULTS

Mutants which do not emit or relay the aggregation signal would be aggregateless or would form very small aggregates. Thirty-two aggregateless mutants were isolated from the standard strain NP14 at survival rates of 1-5 % after -ray irradiation. Various methods were used to find signal- or relay-less mutants among them.

Time-lapse cinematography.

As a first step, cell movements during aggregation stage were analyzed using time-lapse films on NP14 and 5 complete aggregateless mutants isolated. In the standard strain NP14, individual cells showed pulsatile movements toward aggregation centers and such movements as a whole propagated outward from the centers like waves. The intervals of intermittent waves were 7 min at the onset of aggregation and gradually became shorter to 5 or 4 min in later stage. Eventually cells were incorporated into the aggregation streams leading to the centers. These observations were consistent with others (Shaffer, 1962; Gerisch, 1968), and such organized pulsatile cell movements are believed to be primarily ascribed to periodic emissions of aggregation signal from the centers (Robertson et al., 1972; Durston, 1974).

However, the cells of the complete aggregateless

mutants never showed ordered movements even after prolonged incubation, but moved randomly. This suggested that these mutants might be defective in emission of the aggregation signal.

Light-scattering changes of cell suspension.

To ensure that they did not emit the signal, light-scattering of cell suspension was measured. In NP14, when cells were used 6 hr after starvation, periodic decrease of optical density was observed (Fig. 1a). The intermittent decreases occurred at nearly same intervals as the periodic cell movements observed in time-lapse films. This change has been considered to be related to the activity of signaling-cells and probably represents the changes in either cell shape, volume or degree of agglutination in response to the signal (Gerisch & Hess, 1974; Alcantara & Monk, 1974). However, cells of HT41, one of the aggregateless mutants, did not show such a change under the same conditions even after the prolonged incubation (Fig. 1b). Therefore, HT41 seems indeed to be a mutant deficient in signal emission.

Furthermore, another significant defect of HT41 was revealed from the analysis of light-scattering changes caused by cAMP. The response to the nucleotide was quite different between NP14 and the mutant cells. When cAMP was added into aggregation-competent cell suspension, NP14 cells showed two peaks of decrease of optical densi-

ty, fast response and slow response (Fig. 2a), while HT41 cells showed only one peak (Fig. 2b). The response of mutant cells coincided with the fast response of NP14 cells. It is suggested that the fast response may correspond to the response of cells to applied cAMP and that the slow response may be the response of cells to cAMP released from signal-relaying cells which have been stimulated by externally added nucleotide (Gerisch & Hess, 1974).

It is well known that signaling- and relaying-cells are not found in the cell population and that no oscillations in light scattering are observed till at least 4 hr after starvation (Robertson et al., 1972; Gerisch & Hess, 1974). Then, if cAMP is added to cell suspension, only chemotactic response without signal relay is expected to be observed. NP14 cells at about 4 hr starvation were stimulated by the nucleotide and light-scattering changes were recorded. As shown in Fig. 2c, the cells responded to the stimulus but showed only one peak in light-scattering decrease. This peak coincided with fast response of biphasic decrease of aggregation-competent cells.

These results suggest that strain HT41 has defect in a signal relay system.

Cyclic AMP pulses on a cell layer.

Lack of signal relay in HT41 was shown by another additional experiment. Amoebae were freed from bacteria and plated on non-nutrient agar. After they settled, drops of cAMP were administered on a cell layer at intervals of 5 min as an artificial signal for aggregation. Forming the characteristic streams, NP14 cells were attracted toward the source of the signal (Fig. 3a). Although the mutant cells could respond and were attracted, they did not form the streams but gathered centripetally (Fig. 3b). It is well known that local signal relay causes the cells to assemble into the streams in D. discoideum (Shaffer, 1962), and that in the aggregation of D. minutum, which is known for the lack of signal relay, aggregation streams are not formed (Gerisch, 1968). Therefore, it is likely that HT41 has a defect in the signal relay system.

Co-sporulation with NP14 cells.

If mutant strain HT41 has no serious defects other than in signal emission and relay, HT41 cells may aggregate and eventually differentiate into normal spores, when standard strain NP14 cells help them in generating and transmitting the signal in the mixed cell population. This possibility was examined. The mutant cells were

mixed with the standard cells and allowed to develop on non-nutrient agar. After 2 days incubation, spores were collected and examined by plating them clonally. Consequently, aggregateless plaques were found in sufficient amount among fruiting body-forming plaques. Thus, though HT41 cells could neither emit nor relay the signal, it was clear that they possessed the ability to complete the developmental program.

Extracellular cAMP-phosphodiesterase, contact sites A and the effects of cAMP pulses.

Darmon et al. (1975) isolated many aggregateless mutants and classified them into 3 groups by cellophane test. Among them, Group II mutants, which seemed to be quite similar to aggregateless strain HT41, displayed an enhanced chemotactic response to cAMP and developed contact sites A when treated with cAMP pulses (Darmon et al., 1976). Most mutants of this group did not show any substantial increase in cellular phosphodiesterase activity, but when subjected to cAMP pulses they showed a dramatic increase (Darmon et al., 1976). In this experiment, we examined effects of cAMP pulses on the aggregateless mutant HT41. The cells were shaken in the buffer solution with or without the addition of cAMP pulses. Samples were taken at regular intervals, and

cohesive properties of the cells and extracellular phosphodiesterase activity were assayed. NP14 cells became cohesive after 4 hr and formed agglutinates which could not be dispersed by EDTA due to the acquisition of contact sites A, while HT41 cells dis not. But the mutant cells could gain such cohesiveness when imposed by cAMP pulses (Fig. 4). The enzyme activity of NP14 increased after starvation, reaching maximum at 6 hr, whereas such increase was not observed in HT41. However, when cAMP pulses were applied, HT41 cells also accumulated substantial level of phosphodiesterase (Fig. 5).

DISCUSSION

Periodic emission and relay of the aggregation signal may be the most critical events in the cell aggregation of D. discoideum (Loomis, 1975; Newell, 1977). All results obtained here are consistent in respect that strain HT41 is a signalless and relayless mutant.

This is the first report that describes the light-scattering pattern of aggregateless strain. The periodic decrease of light-scattering in wild-type has been considered to be related to the activity of signaling-cells (Gerisch & Hess, 1974). There is evidence that the decrease is always accompanied by the peak of extracellular cAMP concentration (Gerisch & Wick, 1975). Therefore, this procedure was thought to be sufficient to analyze signal emission in developmental mutants. Such periodic decreases were not observed in aggregateless HT41 (Fig. 1b), which was concluded to lack signal emission, together with the result of time-lapse film.

Externally applied cAMP induced a biphasic decrease, fast and slow responses, in light-scattering in NP14 cell suspension (Fig. 2a). The former would correspond to response to external cAMP and the latter would be caused by the endogenous nucleotide released by relaying as suggested by Gerisch & Hess (1974). The reason which

leads to above notion is that time lag between fast response and appearance of slow response corresponds to that required for signal relay reported by Alcantara & Monk (1974), and that slow response is not observed in preaggregative cells of NP14 which are immature for signal relay (Fig. 2c). Besides Wurster et al. (1977) presented the evidence that intracellular cAMP which was released into extracellular space reached maximum concentration just prior to slow response. In the present experiment, externally added cAMP elicited only fast response in HT41 cell suspension (Fig. 2b). Therefore, HT41 was suggested to have defect in signal relay. This was further confirmed by the aggregation pattern induced by artificial signal (Fig. 3b).

In D. discoideum, intercellular signal, cAMP, is thought to be received by its receptors on cell surface, and processed to reach intracellular targets, causing such responses as chemotaxis, induction of synthesis of phosphodiesterase and contact sites A, and transient activation of adenylate cyclase (Newell, 1977; Gerisch et al., 1977). Aggregateless HT41 seems to have functional receptors, since cAMP causes characteristic decrease in light-scattering (Fig. 2b) and cAMP pulses can attract the cells (Fig. 3b), induce accumulation of extracellular phosphodiesterase (Fig. 4) and appearance of contact sites A (Fig. 5) in this mutant. But signal

relay is absent. Therefore, the processing pathway to activate adenylate cyclase seems to be different from those mediating chemotaxis, accumulation of extracellular phosphodiesterase and acquisition of contact sites A. Alternatively, cells may have several types of receptor and that involved in signal relay may be different from those mediating other responses, as suggested by Green & Newell (1977).

Darmon et al. (1977) suggested that their group II aggregateless mutants, which showed enhanced chemotactic responses to cAMP and formed EDTA-resistant contacts when pulsed with cAMP, might be unable to sustain a functional oscillatory system. Although aggregateless HT41 seems to resemble phenotypically to group II mutants, there is one difinite difference between them. HT41 can differentiate into spores when mixed with wild-type cells, whereas group II mutants can not.

The deficiency in signal relay is first proved in this experiment. Concerning the mechanisms of appearance of signaling-cells, it is not clear at present whether these cells exist predetermined in the population or appear as differentiation proceeds (Raman, 1976). As strain HT41 can neither emit nor relay the signal, but can respond to it, it may be suitable to study the mechanisms of appearance of signaling-cells. For example,

a series of small drops containing only one wild-type cell and several hundreds of HT41 cells are plated on non-nutrient agar and allowed to aggregate, as suggested by Raman (1976). If signaling-cells are predetermined, most of such cell populations would fail to aggregate. On the contrary, if all cells of wild-type have the potential to differentiate to them, most populations would aggregate, though the interval which is required for each to aggregate after the deposition may vary.

Also, as strain HT41 can not acquire aggregation-competence by itself but can gain it when stimulated by either artificial or naturally occurring signal, it may be suitable for the study of the differentiating processes leading to the cellular differences in the population and other unsolved mechanisms accompanied with cell aggregation.

SUMMARY

In Dictyostelium discoideum, a developmental mutant, HT41, was isolated. By time-lapse cinematography, this mutant was shown to lack the signal emission. It was proved directly by light-scattering pattern of the cell suspension. By light-scattering, defect of the signal relay was also found. HT41 cells could not form aggregation streams but gathered centripetally, when artificial signals, cAMP pulses, were administered on the cell layer. They could aggregate and differentiate into normal spores, if they were mixed with wild-type cells. Results suggested that HT41 was the mutant which had a defect only in the signal emission and relay system of their developmental program. This kind of mutant would be useful to study the mechanisms of appearance of signal emitting-cells and relaying-cells in cell population during aggregation in the cellular slime mould.

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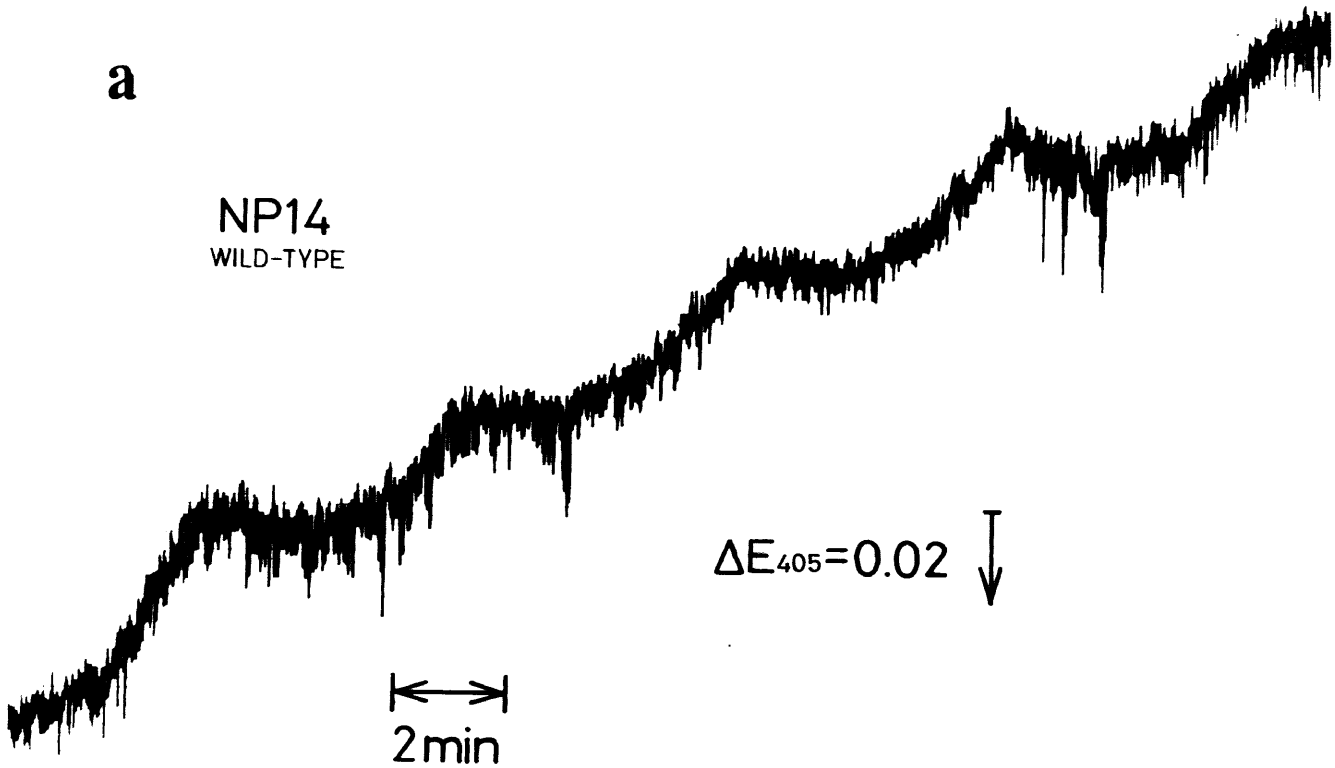
Fig. 1. Light-scattering changes of aggregation-competent cell suspension. After 6 hr starvation at 10^7 cells ml^{-1} in 17 mM phosphate buffer, pH 6.0, cells were concentrated to 3 x 10^7 cells ml^{-1}, bubbled, and monitered at 405 nm in recording photometer. The optical density decreased periodically in standard strain NP14 (1a), but gradually in aggregateless mutant HT41 (1b).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NP14
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ΔE405=0.02
2min

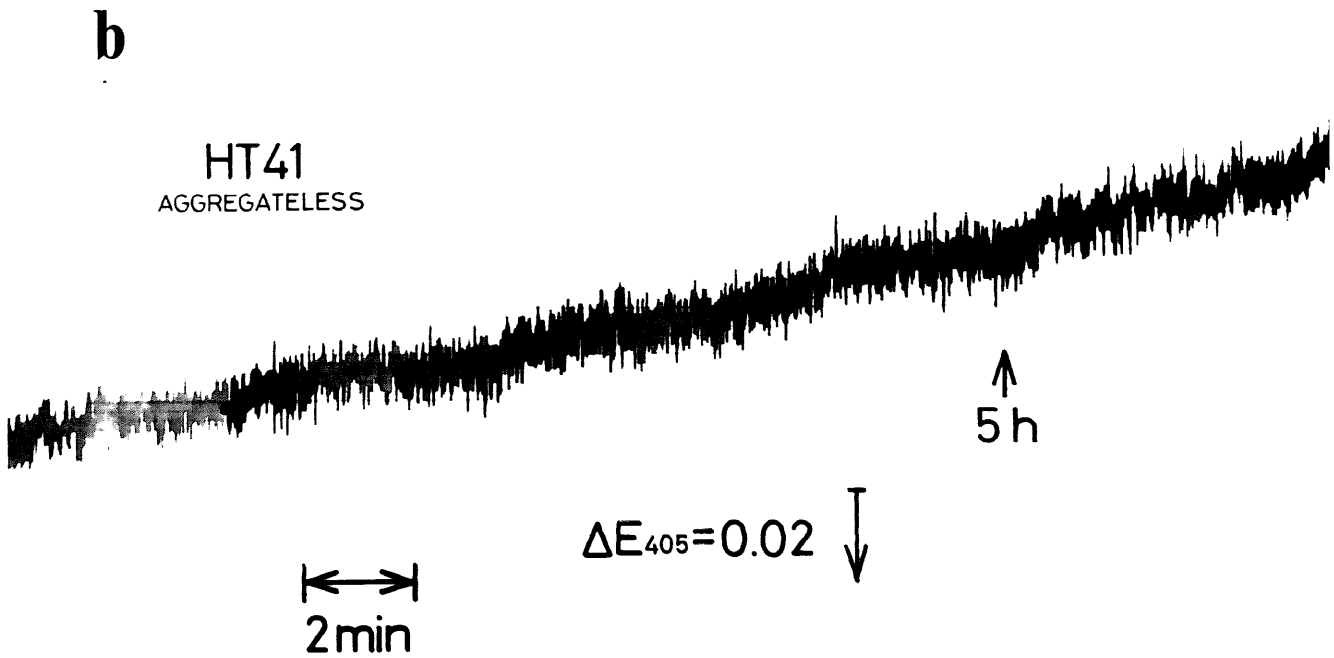
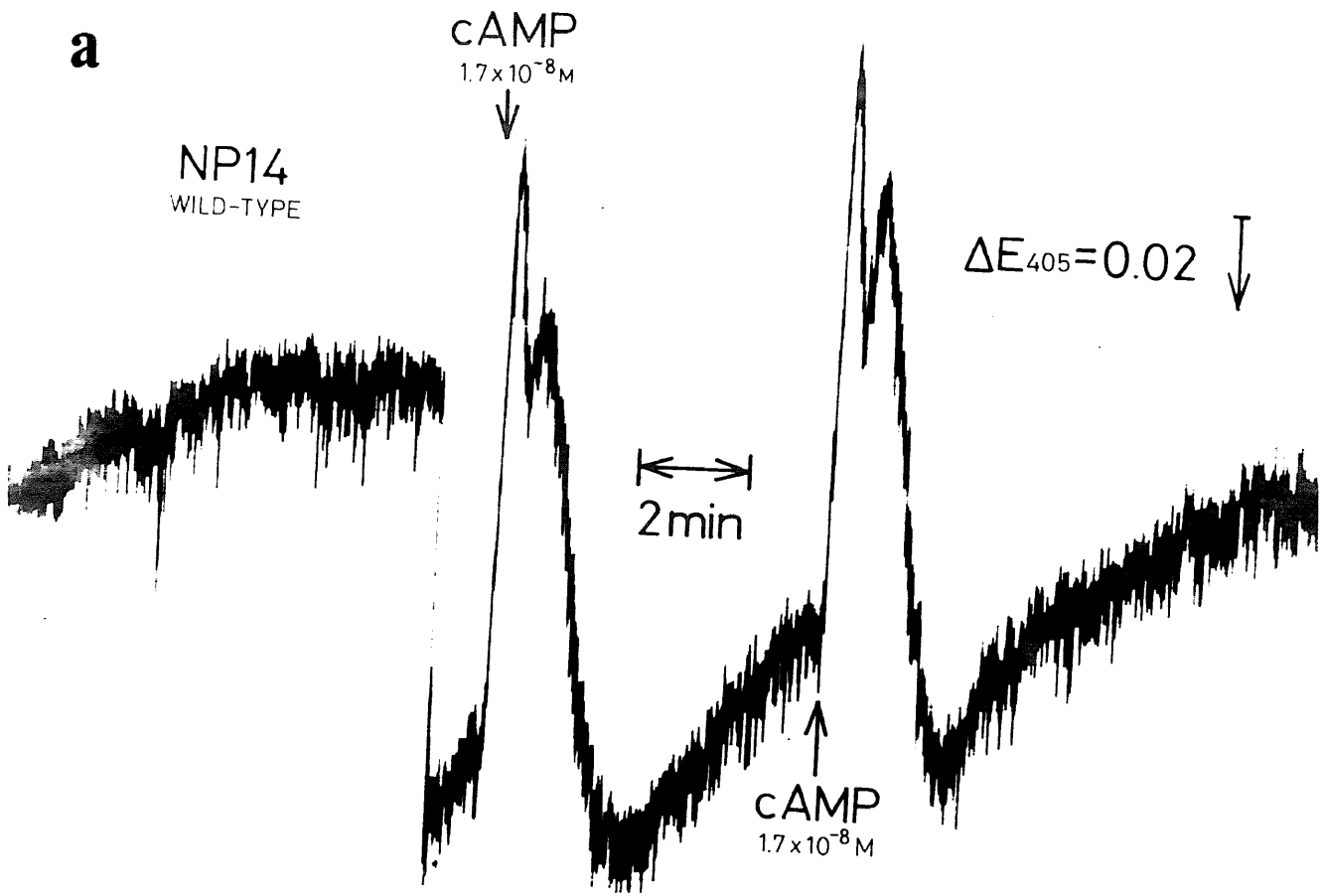

b
HT41
AGGREGATELESS
5h
ΔE405=0.02
2min

Fig. 2. Changes in light-scattering following addition of cAMP. Cyclic AMP were added to 2.5 ml of cell suspensions starved for either 6 hr (2a and 2b) or 4 hr (2c). The arrow indicates the addition of cAMP. The molarities indicated are the final concentrations of added cAMP in the cell suspension. Aggregation-competent cell suspension of standard strain NP14 showed a biphasic decrease of light-scattering after administration of cAMP. Fast response was followed by slow response (2a). Aggregation-competent cell suspension of aggregateless mutant HT41, however, showed only one peak of fast response (2b). Preaggregation cells of NP14 also showed fast response after addition of cAMP (2c).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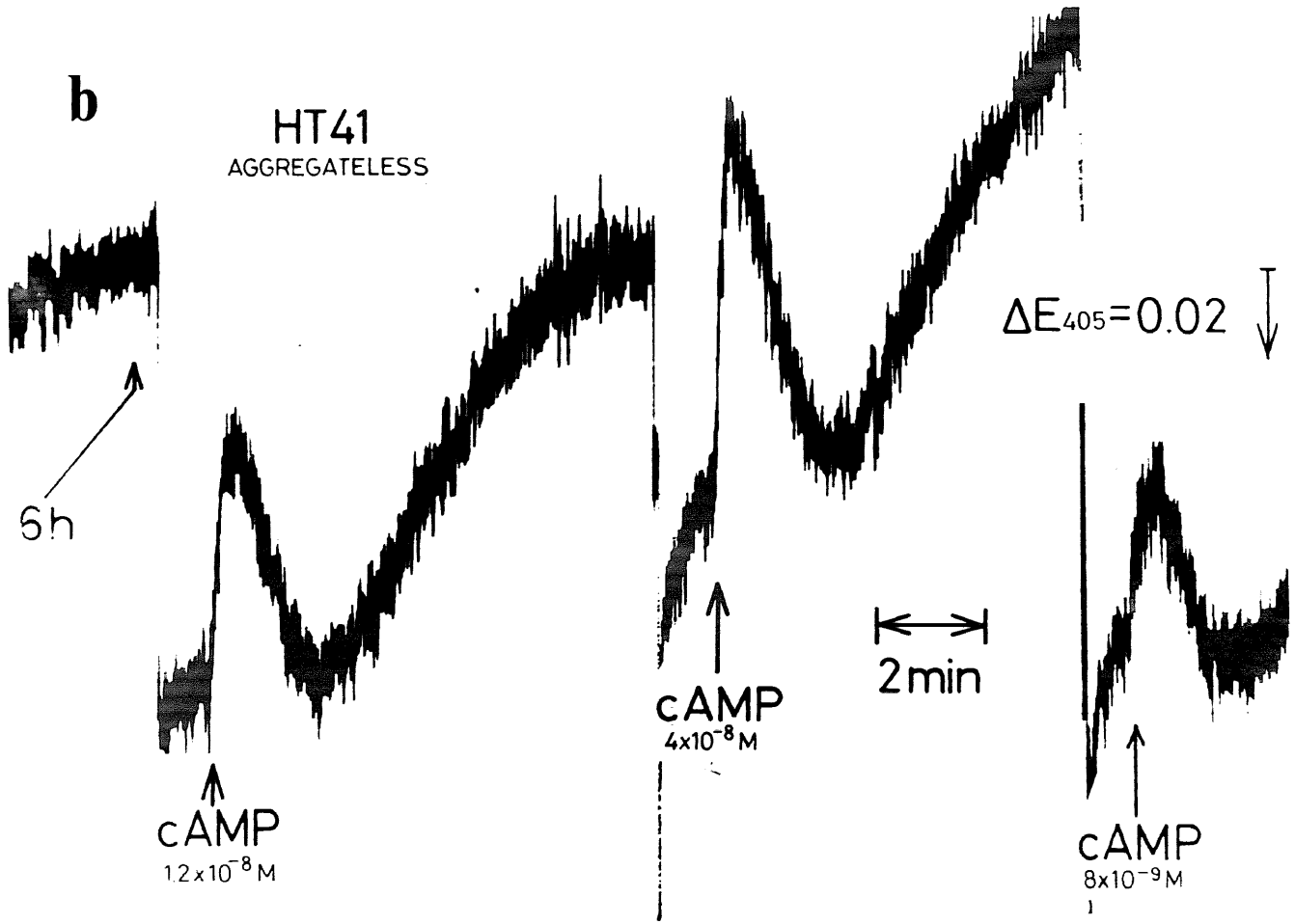
2min
cAMP
1.7 x 10⁻⁸ M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HT41
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6h
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cAMP
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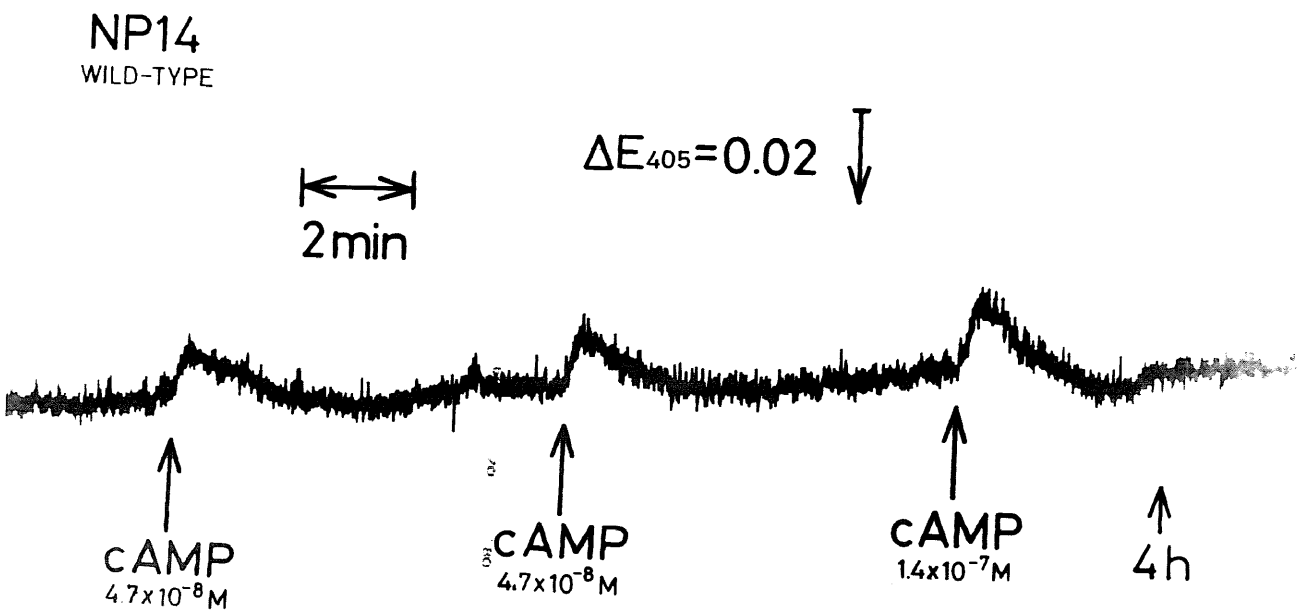
2min
ΔE405=0.02
cAMP
8x10⁻⁹ M

c


NP14
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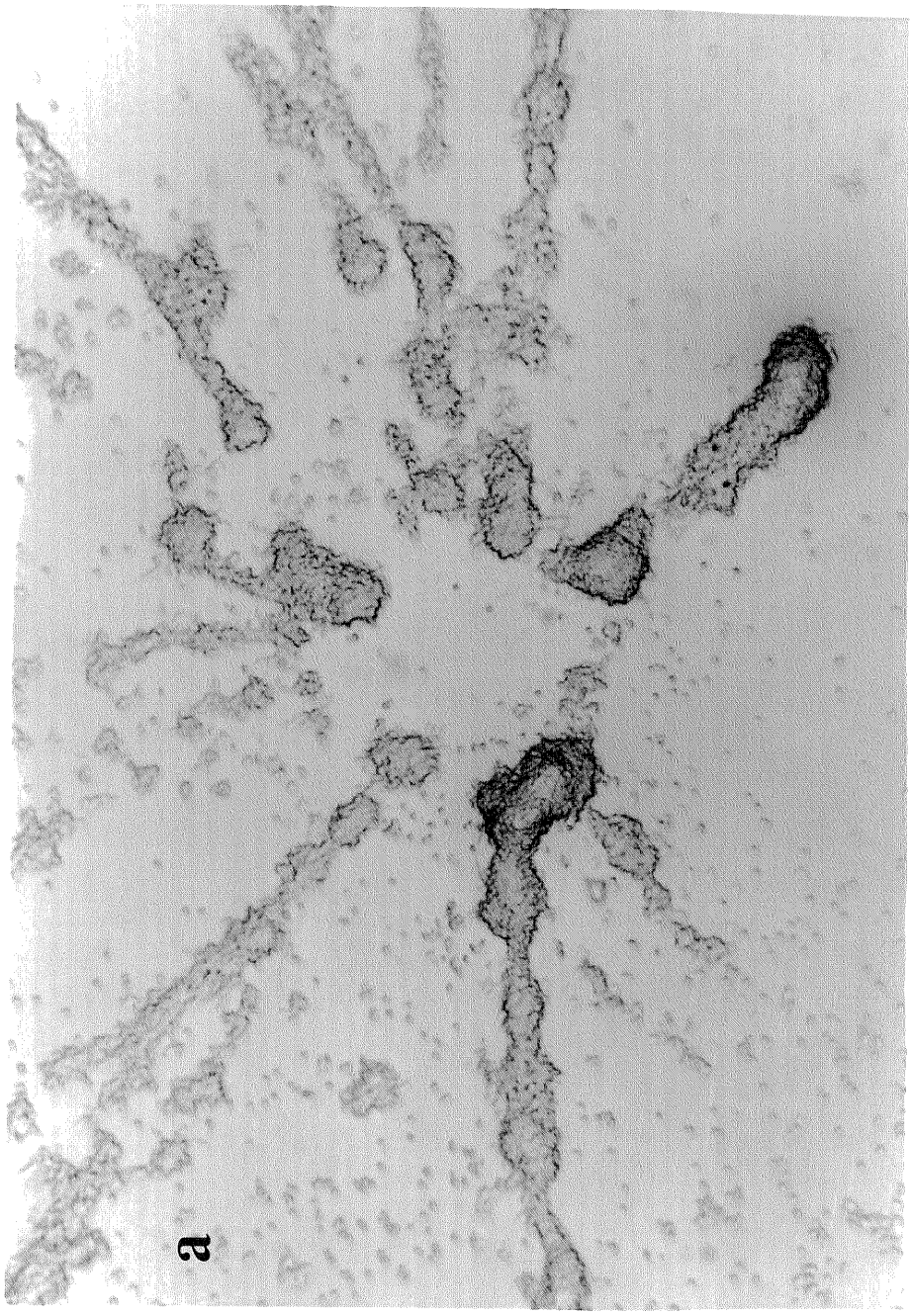
ΔE405=0.02
2min
cAMP
4.7x10-8M
cAMP
4.7x10-8M
cAMP
1.4x10-7M
4h

Fig. 3. Aggregation pattern induced by the addition of cAMP pulses. Amoebae were deposited on non-nutrient agar at a density of 10^7 cells cm^{-2}, and 5 ul of cAMP (10^{-4} M) was added dropwise on a cell layer at intervals of 5 min for 14 hr. NP14 cells formed aggregation streams (3a), but HT41 did not (3b).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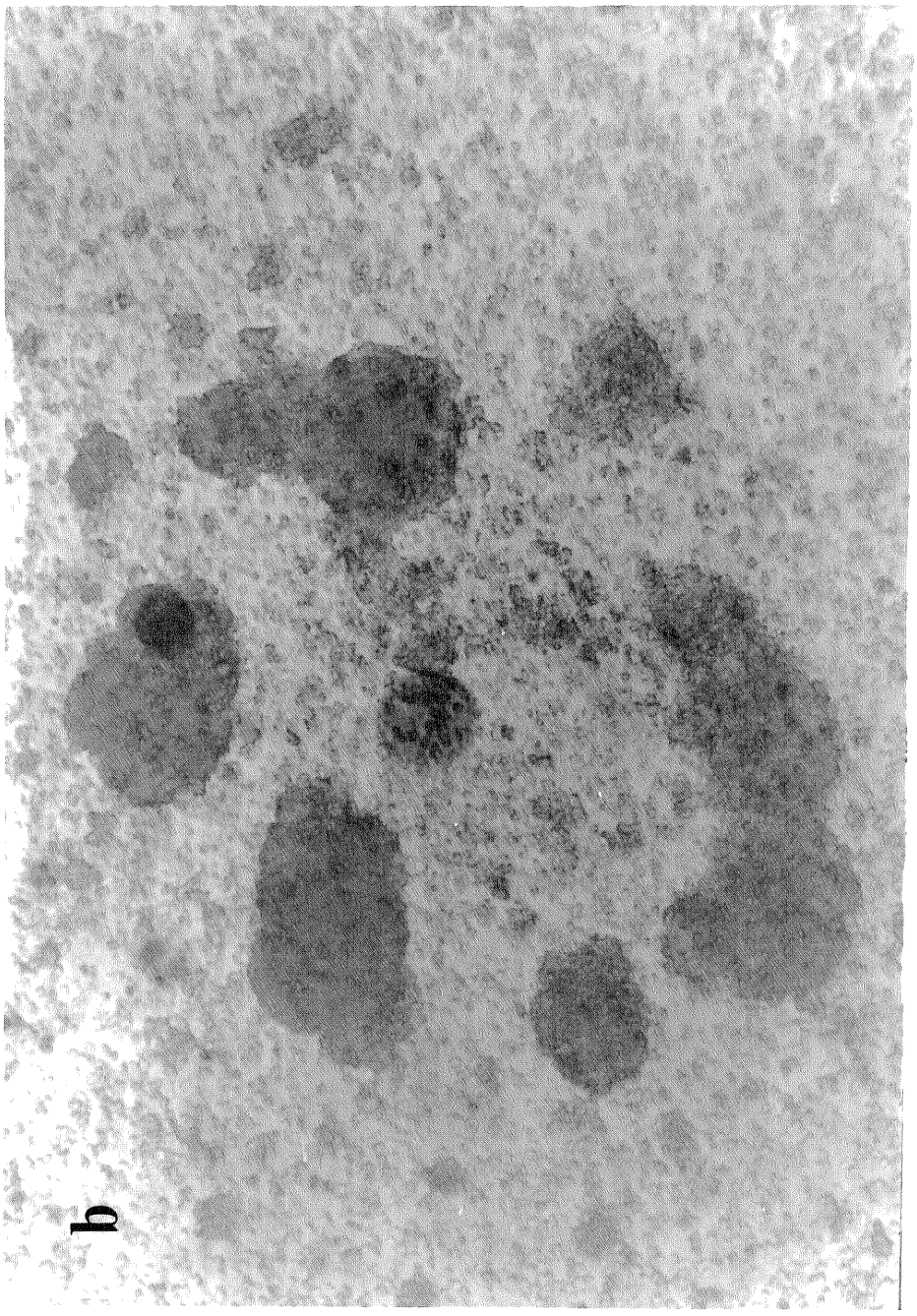
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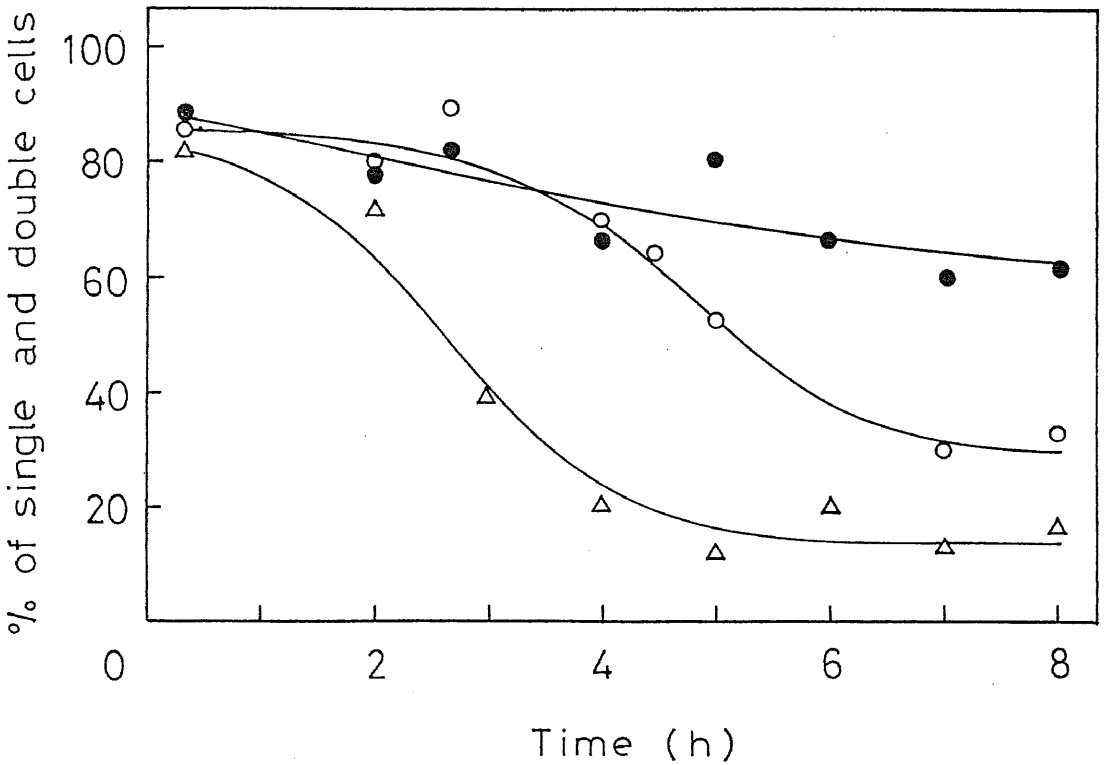
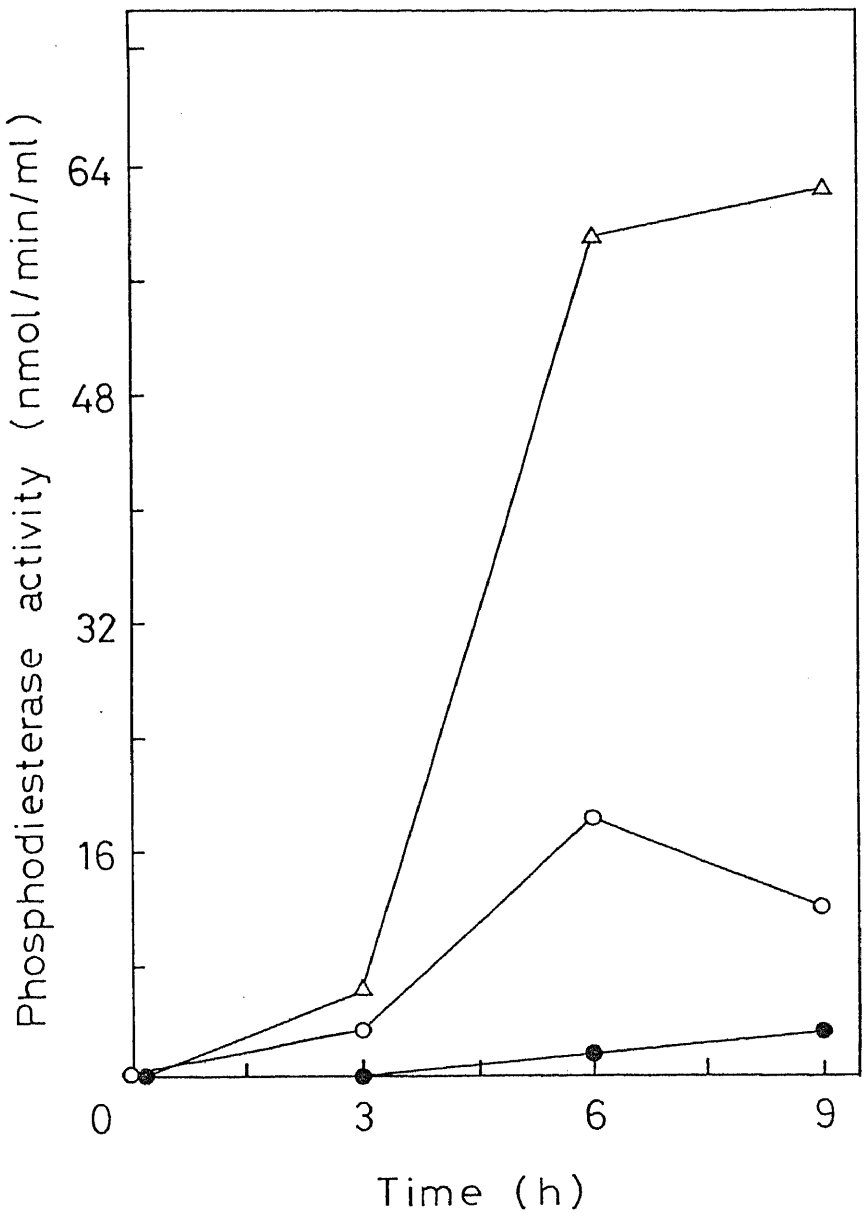

% of single and double cells
100
80
60
40
20
0
2
4
6
8
Time (h)

Fig. 5. Effects of cAMP pulses on extracellular phosphodiesterase activity. Growing cells were washed by centrifugation, suspended in buffer solution at 10^7 cells ml^{-1} and shaken with or without addition of cAMP pulses. At indicated times, aliquots were removed and centrifuged at 3000 rev. min^{-1} for 30 min. After dialysis against 10^{-2} M Tris-HCl buffer, pH 7.5, phosphodiesterase activity was assayed. NP14 without cAMP pulses showed a normal increase of the enzyme activity during the development (o), but HT41 without the pulses did not show a significant increase of the activity (●). When cAMP pulses were administered, however, HT41 showed a dramatic increase (△).


Phosphodiesterase activity (nmol/min/ml)
64
48
32
16
0
3
6
9
Time (h)

PART II

GENETICAL ANALYSIS OF A SIGNAL- AND RELAY-LESS MUTANT, HT41.

INTRODUCTION

It is worthwhile to reveal the regulation mechanism at the gene level as well as the molecular and cellular level. Despite the availability of numerous mutants affecting the development, however, no means of genetic analysis was available until recently in Dictyostelium discoideum. Its life cycle contains no obligately sexual stage. Almost all of the haploid cells retain their individuality throughout the cycle. A complete sexual cycle has been postulated, but it is not clear at present (Filosa & Dengler, 1972; Erdos, Raper & Vogen, 1973; Macinnes & Francis, 1974; Francis, 1975).

But recently this organism has been shown to have a parasexual cycle similar to that of Aspergirus nudulans (Pontecorvo, 1956; Sussman & Sussman, 1963; Loomis & Ashworth, 1968; Sinha & Ashworth, 1969). It is a similar process in principle to that used commonly in somatic cell genetics. It includes the following processes (see Fig. 1). On occasion two haploid cells fuse to form a heterokaryotic cell at a low frequency (10^{-5}-10^{-6}). Then the nuclei undergo karyogamy to give rise to a diploid cell. It propagates indefinitely but sometimes loses chromosomes at a low frequency (10^{-3}-10^{-4}), giving rise to aneuploid and haploid progeny. As loss of chromo-

some occurs at random, genetic exchange takes place. Through this cycle, therefore, genetic analysis is possible.

However, since both diploid formation and segregation occur at low frequency, selective techniques in botn steps are needed for routine genetic analysis. For this purpose, suitable methods were introduced by Loomis (1969) and Katz & Sussman (1972). In their methods the haploid parental strains are given non-allelic mutations to temperature sensitivity for growth and, at the same time, one of them is also marked with a recessive drug-resistant mutation. The rare heterozygous diploids formed by fusion can be selected at a restrictive temperature which does not allow the growth of both haploid parents. If the parents have other genetic markers, dominance relationships and complementation between them can be determined from the phenotype of the diploid. On the other hand, the presence of the recessive drug-resistant marker permits the selection of drug-resistant haploid progeny. The number and phenotype of these segregants with other unselective markers determine the linkage relationship between genetic markers. Since then, various markers have been located on linkage map and six linkage groups have been established so far (Williams, Kessin & Newell, 1974a;

Kessin, Williams & Newell, 1974; Free, Schimke & Loomis, 1976). While cytological evidence shows that the haploid chromosome number of D. discoideum is seven (Wilson, 1953; Brody & Williams, 1974).

The present study has been initiated to elucidate the mechanism of cell aggregation at the gene level. To complete the linkage groups has been another purpose of this study. In this paper, the mutation of aggregateless HT41 was examined for both dominance-recessive relationship to wild-type allele and linkage relationship to other markers.

MATERIALS and METHODS

Chemicals. Proteose peptone and yeast extract were obtained from Difco Laboratory. Cycloheximide and p-fluorophenylalanine were obtained from Wako Pure Chemical Industries, Co. and acriflavin from Tokyo Kasei Industries. Streptomycin sulphate was purchased from Meiji Seika Kaisha, LTD. All other chemicals were analytical grade.

Strains. Used strains were D. discoideum NC-4 (Raper, 1935), NP14 (Williams et al., 1974a), X2 (Williams et al., 1974a) and HT41 (Konno et al., in press). NP14 and X2 were obtained from Dr. P. C. Newell, Oxford University. HT41 was a signalless and relayless mutant isolated from NP14. The characteristics of these strains are detailed in Table 1 and genetic notation used is described in Table 2.

Growth conditions and media. Cells were grown on nutrient agar in association with A. aerogenes at 22 C unless othewise stated (Konno et al., in press). Drugs µsed for the selection of haploid progeny were added to the molten agar to the following concentration in µg per ml : cycloheximide, 500; acriflavin, 100). Methanol was added to dried nutrient agar to the final

concentration of either 3 or 3.5 % (V/V) (Williams et al., 1974a). Para-fluorophenylalanine was added to molten agar at a concentration of 0.1 % (W/V) (Coukell & Roxby, 1977).

Diploid formation. Heterozygous diploid strain was constructed between haploid X2 and HT41 according to the method of Williams, Kessin & Newell (1974b). These haploid strains carried non-allelic growth temperature-sensitive mutations. They were grown separately on nutrient agar with A. aerogenes for approximately 48 hr at 22 C. The amoebae were harvested, washed repeatedly by centrifugation to remove the remaining bacteria, and re-suspended in LPS (Sussman, 1966). Portions of two strains containing 1×10^7 cells/ml were mixed in LPS. The mixture were shaken reciprocally at 120 strokes/min at 22 C for about 12 hours. Under these conditions amoebae formed large clumps of agglutinated cells. After incubation, cells were collected, separated by vortexing and plated on nutrient agar with bacteria at 1×10^5 - 1×10^7 cells per plate. These plates were incubated at restrictive temperature (27 C) till plaques appeared. Haploid and diploid obtained were distinguished by spore size.

Selection of haploid segregants. Haploid segregants were obtained from the diploid by plating amoebae or spores with bacteria on nutrient agar containing either cycloheximide, acriflavin or methanol (Williams et al., 1974a). Alternatively diploid amoebae were cultured in axenic medium which contained streptomycin sulphate (250 µg/ml) to avoid the bacterial growth (Williams et al., 1974b).

The phenotype of the segregants was determined by transfering each of them on nutrient agar previously spread with bacteria.

RESULTS

The mutated gene of aggregateless HT41 was designated as agg-1. To study whether this allele is dominant to wild-type one or not, as well as to assign it to a linkage group previously established, the cells of HT41 and strain X2 were fused to form diploid.

Isolation of diploid and its characteristics.

Diploid was obtained at non-permissive temperature (27 C) which did not allow the growth of parental strains. The diploid strain was designated as DT24. One of the criteria for diploid was spore size. As shown in Fig. 2, the spore of DT24 was larger than those of either X2 or NP14 from which HT41 was derived. The agg-1 allele was found to be recessive since diploid heterozygous for this marker underwent cell aggregation and constructed normal fruiting bodies (Fig. 3). Also, both whi gene of HT41 which causes the formation of white spore and bwn gene of X2 which is responsible for the production of brown pigment were found to be recessive, because cells of DT24 formed yellow spores and did not produce brown pigment (Fig. 3). It was consistent with Katz & Sussman (1972).

Assignment to a linkage group.

In order to examine the linkage relationship between agg-1 gene and other markers, haploid segregants were screened from DT24 using various selection media.

At first, DT24 cells were plated on nutrient agar containing both cycloheximide (500 μg/ml) and methanol (3 %). After 10 days of incubation, resistant clones which had both cyc and acrA genes, appeared in the bacterial lawn at frequencies of 10^{-5} - 10^{-6}. The segregants obtained were scored for the phenotypes of unselected markers. As shown in Table 3, almost segregants were aggregateless. Then, agg-1 gene was suggested to be located on either linkage group I (cyc gene) or II (acrA gene). It was further confirmed by plating the diploid cells on nutrient agar containing both cycloheximide and acriflavin (100 μg/ml). In this case, however, the growth of cells were considerably slower than that on medium containing both cycloheximide and methanol. It was shown to be solely ascribed to the presence of acriflavin (data not shown). Nevertheless, the major class of segregants was aggregateless phenotype (Table 4). Therefore, it is probable that agg-1 gene is located on either linkage group I or II.

Linkage relationship between agg-1 and cyc gene.

As a next step, it was examined whether agg-1 gene was linked to cyc gene. DT24 cells were plated on nutrient agar containing only cycloheximide. It was expected that all segregants isolated would be aggregateless if agg-1 gene was linked to cyc gene. In this case, however, the segregants having the ability to aggregate were obtained nearly twice as many as aggregateless ones (Table 5). Then, agg-1 gene seemed not to be linked to cyc gene. As Rothman & Alexander (1975) suggested that one drug selection resulted in the enrichment of the fraction of diploid segregants which acquired drug resistance due to the mitotic crossing over, however, fruiting body-forming segregants were suspected of diploid.

Then, after DT24 cells had been grown on nutrient agar containing p-fluorophenylalanine (1 %, W/V) which was known to promote haploidization (Coukell & Roxby, 1977), they were plated on cycloheximide-containing agar. This procedure gave rise to a rather high recovery of resistant segregants. As shown in Table 6, though a partial increase of the fraction of aggregateless segregants was observed, there was still a sufficient number of fruiting body-forming segregants. Therefore, this class of segregants was considered to be haploid. Then it is probable that agg-1 gene is independent to linkage group I.

Linkage relationship between *agg-1* and *acrA* gene.

As indicated above, diploid cells were precultured on nutrient agar containing p-fluorophenylalanine. Methanol-resistant clones were isolated from these cells. In this case, almost resistant clones were aggregateless (Table 7). Therefore, *agg-1* gene seemed to be linked to *acrA* gene. The fruiting body-forming clones were probably diploid which arose from mitotic crossing over.

The relation of *agg-1* gene to the genes which confer the ability to grow axenically.

To confirm that *agg-1* gene was located on linkage group II, segregants having the ability to grow axenically were selected by shaking DT24 cells in axenic medium. This ability is a recessive character and is conferred by both genes *axeA* (linkage group II) and *axeB* (linkage group III) (Williams *et al.*, 1974a). In this experiment, it was expected that all of axenic segregants would be fruiting body-forming if *agg-1* gene was linked to linkage group II. Because it resulted in that *agg-1* and *axeA* genes were mapped on the different homologues. As shown in Table 8, this was the case. Most segregants isolated were able to perform aggregation. Therefore, it is likely that *agg-1* gene is linked to $axeA^{+}$ gene, wild-type allele to *axeA*, on linkage group II.

DISCUSSION

The agg-1 mutation of aggregateless HT41 is located on linkage group II. Furthermore, the relative position of agg-1 gene to other makers on linkage group II may be deduced from the appearance of mitotic diploid. On linkage group II, the order of 2 markers has been established as follows (Mosses, Williams & Newell, 1975).

Centromere----whi----acrA.

If agg-1 gene is located distal to acrA gene, the selection for acrA gene should result in that all segregants obtained are aggregateless irrespective of haploid or diploid. But this was not the case (Table 3, 4 and 7). Therefore agg-1 gene is located proximal to acrA gene. Then, the order of the genes, centromere, whi and agg-1, is discussed. If agg-1 gene is located distal to whi gene as shown in model 1 of Fig. 4, any crossing over at the positions of I, II or III do not produce fruiting body-forming segregants with white spores. On the contrary, if agg-1 gene is located on proximal to whi gene as indicated in model 2, mitotic crossing over at the position II' gives rise to fruiting body-producing diploid with white spores and similar haploid if haploidization is followed. Experimental results shown in Table 3, 4 and 7 are consistent with model 2. Therefore the order

of centromere--agg-1--whi--acrA may be established.

The behabior of bwn gene which is responsible for the production of brown pigment was curious. It has been mapped on linkage group IV, and therefore is independent to cyc gene (linkage group I) and acrA gene (linkage group II) (Katz & Sussman, 1972; Williams et al., 1974a). However in our experiment, independent segregation of bwn gene from above two genes was not observed (Table 3, 5 and 7). Even when the segregation of bwn gene occurred, the number of brown segregants was much fewer than expected (Table 4 and 6). These results may suggest some connection between the genes of bwn, cyc and acrA. However this explanation is incompatible with the result shown in Table 8. Axenic selection, which is equivalent to the selection for $acrA^+$ gene, also resulted in few, instead of abundant, recovery of brown segregants. Thus it is more considerable that the segregation between bwn^+ and bwn gene did not occur in our experiments. In non-brown segregants there may be sufficient number of segregants having genotype of (bwn^+/bwn). However, this explanation is also incompatible with Rothman & Alexander (1975) who suggested that double drug-selection resulted in the recovery of haploid at nearly 100 %. Even when double drugs were used for selection, however, we could obtain only

few brown segregants (Table 3 and 4). Though the slow growth of brown clone is partly responsible for a few recovery of brown segregants because selection of the segregant is tend to select the rapid grower against the slower (Wright, Williams & Newell, 1977), nondisjunction of the homologues carrying bwn^{+} and bwn gene respectively may explain more easily our results. As in genetical analysis, collection of complete haploids is required, some improvements in selective procedure are needed for the routine use of parasexual system.

Aggregateless HT41 seems to have a single mutation since haploid segregants were either aggregateless or fruiting body-former. None of them showed aberrations in development other than in aggregation. Though there is some possibility of other mutation closely linked to agg-1 mutation, it is not likely since HT41 had isolated at relatively high rate of survival after mutagenesis.

Coukell & Roxby (1977) analyzed linkage relationship of 28 aggregateless mutations using similar procedure described here and found that as many as 16 mutations among them falled in linkage group II. Aggregateless HT41 also has the mutation in linkage group II. Though it is not certain whether HT41 resembles to some of their group II mutants, these results suggest that the genes involved in cell aggregation may be clustered

on linkage group II and that they may be susceptible to mutation.

Genetical analysis is useful for the genetical study of mechanism of cell aggregation but also may be used for the typing of the aggregateless mutants. As these mutants have not remarkable differences, it is extremely difficult to discriminate them one another phenotypically. It may convenient to analyze them genetically prior to other biochemical or physiological analysis. This may help investigators by saving them from time-consuming and laborious work.

SUMMARY

The mutation of aggregateless HT41 which has the defect in signal emission and relay system was studied using parasexual system. The mutated gene was designated as agg-1. Heterozygous diploid (DT24) for this marker was formed between HT41 and strain X2 which undergoes cell aggregation normally. As DT24 cells performed cell aggregation, agg-1 mutation was found to be recessive. To study the linkage relationship of agg-1 gene to other marker genes, haploid segregants were isolated using various selection media. Gene agg-1 was mapped on linkage group II on which acrA and whi genes had been previously located. The relative order of these 3 genes on linkage group II was discussed. The problem involved in parasexual genetics was also discussed.

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Table 1. Genotypes and phenotypes of the haploid strains of D. discoideum used.

	Genotype						Phenotype*						
Strain	whi	bwn	tsg	cyc	acr	axe	Cy	Ac	Sp. Col.		Pig.	G. Res.	Ax.
NC-4	-	-	-	-	-	-	S	S	Y		NB	G	NG
NP14	whi	-	D	A	A	-	R	R	W		NB	NG	NG
X2	-	bwn	A	-	-	A,B	S	S	Y		B	NG	G

Genotypes are detailed in full in Table 2.

* (Cy) cycloheximide; (Ac) acriflavin; (Sp. Col.) spore color; (Pig.) Pigmentation; (G. Res) growth at restrictive temperature, 27 C; (Ax.) axenic medium; (S) sensitive; (R) resistant; (Y) yellow; (W) white; (NB) not brown; (B) brown; (G) growth; (NG) not growth.

Table 2. Genetic notation and linkage groups in D. discoideum.

Linkage group	Gene symbol	Mutant phenotype	Wild-type phenotype
I	cyc	able to grow in the presence of cycloheximide (500μg/ml)	unable to grow in the presence of cycloheximide (500μg/ml)
II	whi	white spore	yellow spore
	tsgD	unable to grow at 27 C	able to grow at 27 C
	axeA	able to grow in axenic medium (provided that axeB is also present)	unable to grow in axenic medium
	acrA	able to grow in the presence of acriflavin (100μg/ml) or methanol (2%)	unable to grow in the presence of either acriflavin or methanol
III	tsgA	unable to grow at 27 C	able to grow at 27 C
	axeB	able to grow in axenic medium (provided that axeA is also present)	unable to grow in axenic medium
IV	bwn	brown pigment production	without pigmentation

Table 3 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc / +	whi / +	tsgD / +	+ / axeA	acrA / +	+ / axeB	+ / tsgA	+ / bwn	agg-1 / +
SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER					
CYCLOHEXIMIDE (500ug/ml) and METHANOL (3%)	+	Y	-	1	(0.7%)				
	+	W	-	2	(1.4%)				
	-		-	137	(97.9%)				
				140					

Table 4 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc / +	whi / +	tsgD / +	+ / axeA	acrA / +	+ / axeB	+ / tsgA	+ / bwn	agg-1 / +
SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER					
CYCLOHEXIMIDE (500ug/ml) and ACRIFLAVIN (100ug/ml)	+	Y	-	7	(5.2%)				
	+/-	Y/-	-	7	(5.2%)				
	+	W	-	2	(1.5%)				
	-		-	115	(85.8%)				
	-		B	3	(2.2%)				
				134					

Table 5 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc / +	whi / +	tsgD / +	+ / axeA	acrA / +	+ / axeB	+ / tsgA	+ / bwn	agg-1 / +

SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER	
CYCLOHEXIMIDE (500ug/ml)	+	Y	-	78	(59.5%)
	+	W	-	6	(4.6%)
	-		-	47	(35.9%)
				131	

Table 6 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc	whi	tsgD	+	acrA	+	+	+	agg-1
	+	+	+	axeA	+	axeB	tsgA	bwn	+

SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER
CYCLOHEXIMIDE (500ug/ml)	+	Y	-	60 (52.6%)
	+	Y	B	1 (0.9%)
	+	W	-	3 (2.6%)
	+	W	B	2 (1.8%)
	-		-	47 (41.2%)
	-		B	1 (0.9%)
				114

Table 7 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc	whi	tsgD	+	acrA	+	+	+	agg-1
	+	+	+	axeA	+	axeB	tsgA	bwn	+

SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER	
METHANOL (3.5%)	+	Y	-	4	(2.9%)
	+	W	-	7	(5.0%)
	-		-	128	(92.1%)
				139	

Table 8 LINKAGE ANALYSIS OF agg-1

DIPLOID DT24	cyc	whi	tsgD	+	acrA	+	+	+	agg-1
	+	+	+	axeA	+	axeB	tsgA	bwn	+

SELECTION	AGGREGATION	SPORE COLOR	PIGMENT	NUMBER	
EXPT. 1	+	Y	-	114	(89.1%)
	+	W	-	13	(10.2%)
	-		-	1	(0.8%)
AXENIC MEDIUM				128	
EXPT. 2	+	Y	-	150	(87.7%)
	+	Y	B	1	(0.6%)
	+	W	-	19	(11.1%)
	-		B	1	(0.6%)
				171	

Fig. 1. Parasexual cycle in D. discoideum. Haploid strains carrying independent genetic markers, a and b, form diploids at a low frequency. Lines represent individual chromosomes. The diploid propagates but also loses chromosomes at a low frequency giving rise to recombinant aneuploid and haploid progeny.

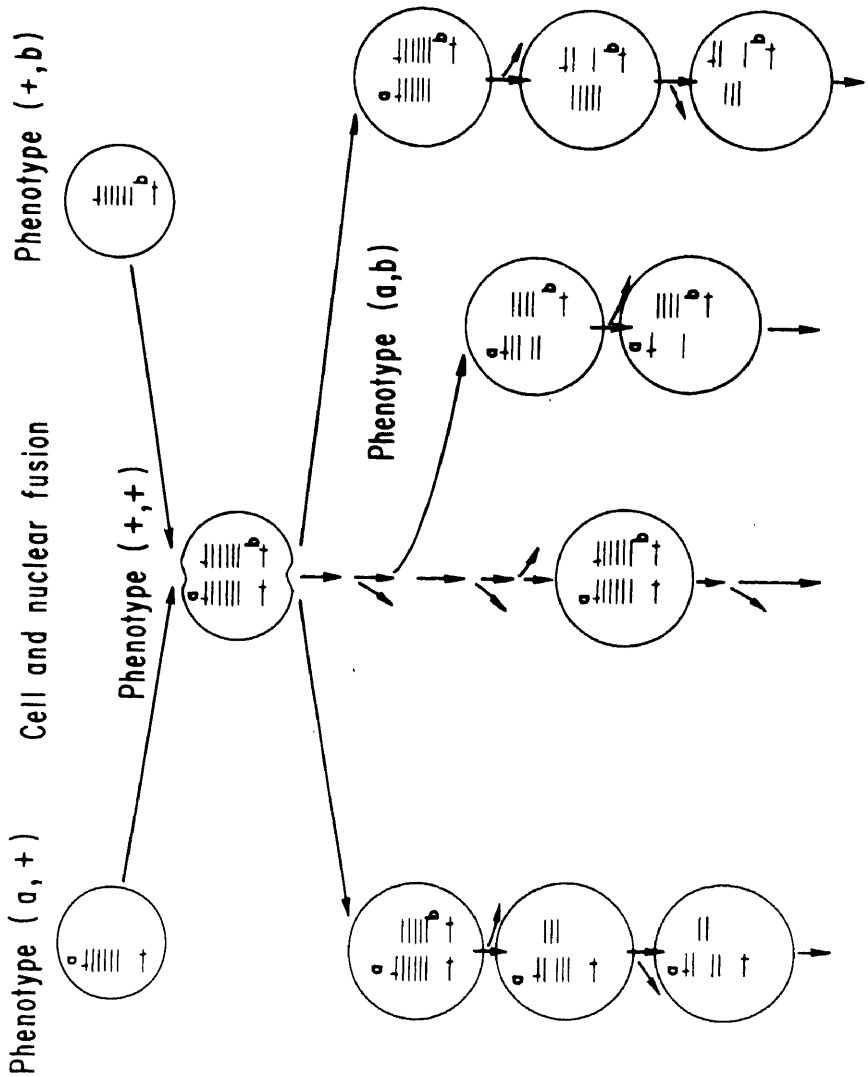

Phenotype (+,b)
Phenotype (a,b)
Cell and nuclear fusion
Phenotype (+,+)
Phenotype (a,+)

Fig. 2. Spore size and shape. Parental haploid strain X2 and NP14 from which aggregateless HT41 was isolated. Diploid strain DT24.

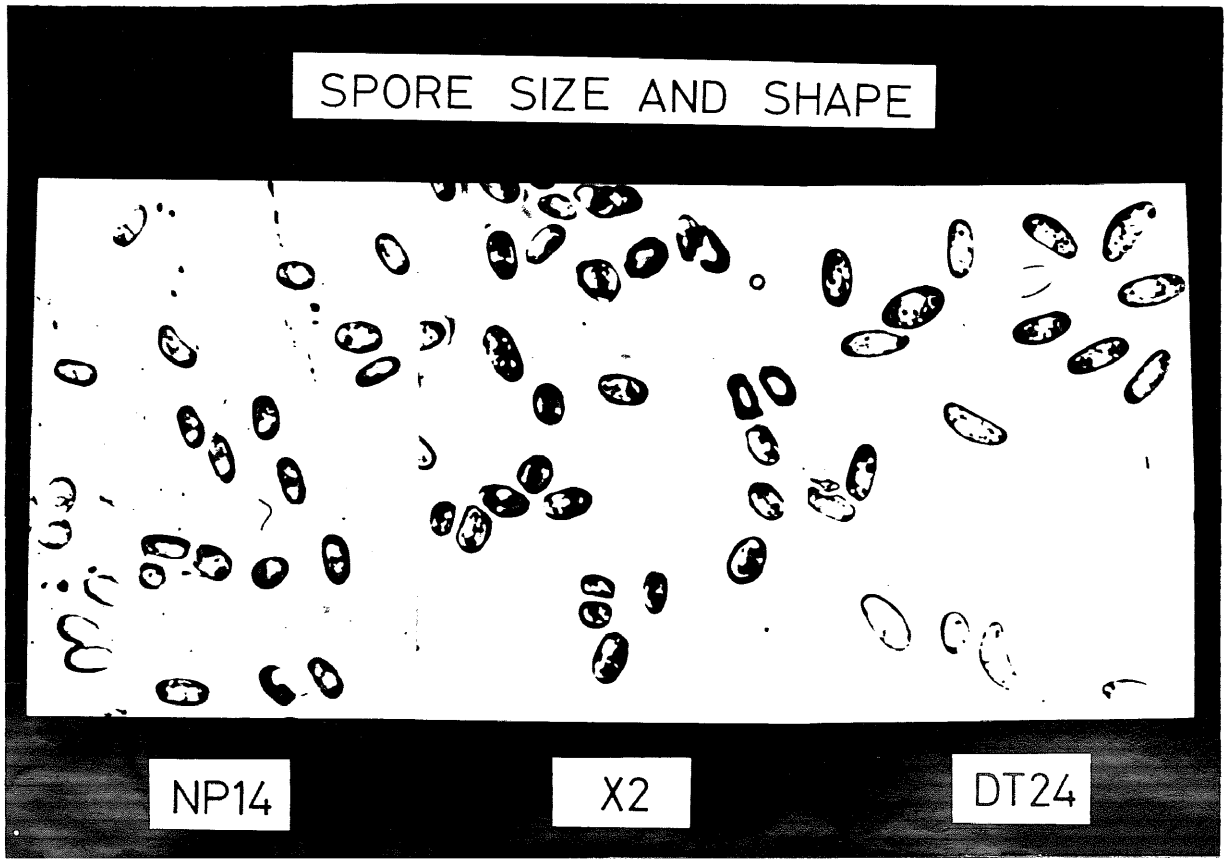
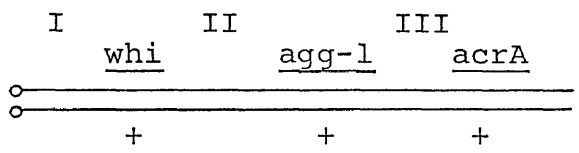

SPORE SIZE AND SHAPE
NP14
X2
DT24

Fig. 3. Fruiting body, spore color and pigment production. Strain NP14 with white spore and without brown pigment production (upper right). Strain X2 with yellow spore and brown pigment production (upper left). Diploid strain DT24 with yellow spore and without pigment production (lower).

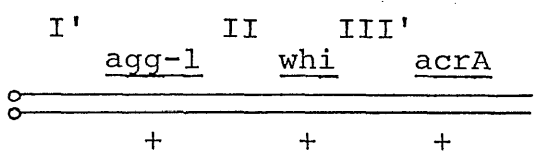
Fig. 4. Tentative gene order of linkage group II and diploids which arise from mitotic crossing over. Genotype and phenotype of the diploid derived from crossing over at the positions of I, II and III are shown. Phenotype is enclosed in parenthesis. Agg^- : aggregateless. Agg^+ : normal aggregation. Y : yellow spore. W : white spore. (Y) and (W) : unexpressed spore color due to aggregateless phenotype.

MODEL 1



crossing-over position	Genotype	Phenotype
I	whi agg-1 acrA / whi agg-1 acrA	Agg^- (W)
II	whi agg-1 acrA / + agg-1 acrA	Agg^- (Y)
III	whi agg-1 acrA / + + acrA	Agg+ Y

MODEL 2



crossing-over position	Genotype	Phenotype
I'.	agg-1 whi acrA / agg-1 whi acrA	Agg^- (W)
II'	agg-1 whi acrA / + whi acrA	Agg^+ W
III'	agg-1 whi acrA / + + acrA	Agg^+ Y

PART III

ANALYSIS OF BIOCHEMICAL PATHWAY IN DEVELOPMENT OF CELLULAR SLIME MOLD, *Dictyostelium discoideum*.

INTRODUCTION

It is well known that cyclic AMP (cAMP) plays important roles in the development and differentiation of the cellular slime mold Dictyostelium discoideum. First, it acts as a signal for aggregation of myxamoebae (Konijn et al., 1967; Robertson, Drage & Cohen, 1972). Second, it accelerates the synthesis of cAMP-phosphodiesterase and of contact sites A which mediate cell adhesion (Klein & Darmon, 1977; Gerisch et al., 1975). At the same time, it inhibits the synthesis of an extracellular phosphodiesterase inhibitor (Klein & Darmon, 1977). Third, it is assumed to be involved in the determination of developmental pathway (Sussman & Schindler, 1978), and acts to form the polarity of slug (Nestle & Sussman, 1972). Furthermore, it induces the differentiation of stalk cells and also is involved in prespore differentiation (Bonner, 1970; Kay, Garrod & Tilly, 1978).

Activities of many enzymes and some membrane proteins are known to appear or disappear at the specific stages during developmental course (Loomis, 1975). Such biochemical activities have been studied by several investigators (see Loomis, 1975), however, the mechanisms causing such changes have not been understood. It is barely known how each change in enzyme activities or

membrane proteins relates to other biochemical events in cells. The developmental process could be either an array of successive reactions or multiple arrays of these reactions. In the latter case, each array of reactions could be either independent or mutually dependent each other.

We previously isolated an aggregateless mutant HT41 which could neither emit nor relay cAMP pulses (Konno *et al.*, in press). This mutant seems to be useful for analyzing some of the causal relations among biochemical events during the early developmental processes. In the present experiments, we examined effects of externally added cAMP pulses on six developmentally regulated enzymes and one membrane protein in the mutant HT41 and the strain NP14 as a wild-type. The results revealed some inter-relations among the changes of these enzyme activities and the appearance of membrane protein during the developmental course of *D. discoideum*.

MATERIALS and METHODS

Chemicals. Para-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-α-D-mannopyranoside, pyridoxal phosphate, and adenosine 3', 5'-cyclic phosphate were purchased from P-L Biochemicals Inc. Para-nitrophenyl-N-acetyl-β-D-glucosaminide, glucose-6-phosphate, phosphoenol pyruvate, and bovine serum albumine were obtained from Sigma. Alpha-Ketoglutarate was a product of Wako Pure Chemicals, Japan. Uridine diphosphoglucose was purchased from Kyowa Hakko Kogyo, Japan. Pyruvate kinase and dinitrophenyl hydazine were obtained from Oriental Yeast Co., Japan and Daiichi Chemicals, Japan, respectively. All other chemicals were of reagent grade.

Strains and culture conditions. Strains used were D. discoideum NP14 and HT41. NP14 was a gift from Dr. P. C. Newell, Oxford Univ. and used as the standard strain. HT41 was an aggregateless strain which had been isolated from NP14 after γ-ray irradiation. It can neither emit nor relay the aggregation signal, cAMP (Konno et al., in press). The cells were grown in association with Aerobacter aerogenes on nutrient agar.

Conditions for development. Growing cells were

harvested using cold Bonner's salt solution (BSS) (Bonner, 1947), centrifuged 4 times to remove bacteria and resuspended in BSS. Cells were plated on non-nutrient agar at 1 x 10^7 cells/cm^2 and incubated at 22 C as described by Sussman (1966). At intervals, they were collected and centrifuged at 2,000 rpm for 3 min. The pellets were frozen at -20 C for subsequent enzyme assays. In another experiment, cells were suspended in 200 ml BSS at 1 x 10^7 cells/ml in an 1 liter flask and shaken reciprocally at 70 strokes/min at 22 C. To study effect of cAMP pulses, 10 µl of the nucleotide solution was added dropwise into cell suspension at intervals of 5 min from 1 or 2 hr, after the initiation of shaking, up to 24 hr. The final concentration of cAMP was 10^{-7} M. At intervals, samples were removed and centrifuged. The pellets were frozen for enzyme assay.

Enzyme assays. Frozen pellets were thawed by the addition of distilled water and lysed by sonication for 30 sec at 25 W with a Tomy sonifier equipped with a micro-tip (Tomy Seiko Co.). Enzyme assays were performed immediately after preparation of the extracts. β-Glucosidase (EC 3. 2. 1. 23) was assayed according to the procedure of Coston & Loomis (1969), α-mannosidase (EC 3. 2. 1. 30) of Loomis (1970), tyrosine transaminase

(EC 2. 6. 1. 5) of Pong & Loomis (1971) and trehalose 6-phosphate synthetase (EC 2. 3. 1. 15) of Roth & Sussman (1968). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. A unit of enzyme activity was defined as that amount which liberated 1 nmol of product / min. Specific activity was expressed as units/mg of protein.

For the assays of extracellular cAMP-phosphodiesterase and EDTA-resistant cell contacts, cells were shaken in suspension with or without addition of cAMP as described above. At intervals, samples were removed. Aliquots were centrifuged at 3,000 rpm for 30 min and the supernatants were dialyzed overnight against 10 mM Tris-HCl buffer, pH 7.5. Phosphodiesterase activity was assayed according to the method of Yanagisawa et al. (1974). To another aliquots EDTA was added at a final concentration of 10^{-2} M. After shaking to dissociate possible agglutinates, the number of total cells and dissociated cells was counted with a haemocytometer. The proportion of single and double cells was calculated.

RESULTS

HT41 was an aggregateless strain which could neither emit nor relay the aggregation signal, cAMP. In HT41 and wild-type NP14 as a standard, changes of activities of six enzymes and one membrane protein during development and effects of cAMP pulses on them were examined. Cells were shaken in sispension and cAMP were applied at intervals of 5 min at a final concentration of 10^{-7} M.

At first three enzymes whose activities changed soon after the initiation of development were examined.

ß-Glucosidase. Changes in the specific activity of ß-glucosidase during early developmental phase were examined and resut was shown in Fig. 1. In wild-type NP14, ß-glucosidase activity began to decrease soon after the initiation of development. The decrease became slow after about 6 hr and gradual decrease continued to 12 hr. The enzyme activity decreased throughout the early developmental phase. Even when cAMP pulses were applied, the decrease in enzyme activity was not affected at all. In aggregateless HT41, though enzyme activity of growing cells was somewhat higher than that of NP14, it decreased in the same way as NP14 during developmental stages. Also, the decrease was not altered by the addition of cAMP pulses.

α-Mannosidase. α-Mannosidase activity of NP14 began to increase also soon after the initiation of development. Accumulation of enzyme continued up to 9 hr and became slow thereafter. When the cells were pulsed with cAMP, the rate of enzyme accumulation increased and a maximum activity was observed at 9 Hr. The maximum activity in the presence of cAMP was higher than the peak activity of the control. The activity declined markedly thereafter. In aggregateless HT41, accumulation of α-mannosidase also occurred immediately after starvation, and reached the plateau at 8 hr. The highest activity was about 70 per cent of the peak level of wild-type strain. When cAMP pulses were applied, accumulation of enzyme also stimulated in HT41. The peak activity was higher than that of unpulsed cells. However, the peak activity of pulsed cells remained constant instead of declining which was observed in wild-type cells (Fig. 2).

N-Acetylglucosaminidase. Following the onset of development, N-acetylglucosaminidase activity of NP14 began to increase, reaching a small plateau at about 9 - 15 hr, and subsequently increased gradually. The enzyme accumulation of NP14 was completely inhibited 2 - 3 hours after the commencement of pulsation and enzyme activity remained at low level throughout. The enzyme accumulation did not occur in HT41 even after the prolonged incubation.

Cyclic AMP pulses did not alter the enzyme accumulation of HT41 (Fig. 3).

Next, one enzyme and one membrane protein which have been shown to be influenced by cAMP pulses were examined.

Extracellular cAMP-phosphodiesterase. Extracellular cAMP-phosphodiesterase activity began to increase 2 - 3 hr after starvation in NP14, reaching maximum at about 6 hr and declining thereafter. When cAMP pulses were applied, the enzyme accumulation was stimulated remarkably. The peak activity was about 4 times higher than that of unpulsed cells. On the other hand, the enzyme accumulation of HT41 was again different from that of NP14. Accumulation was not observed in HT41. However, in contrast to N-acetylglucosaminidase, accumulation of extracellular phosphodiesterase was induced by cAMP pulses (Fig. 4). The effect of cAMP pulses on the accumulation of this enzyme was consistent with previous observations (Klein & Darmon, 1977; Juliani & Klein, 1978).

Contact sites A. Cell contacts mediated by contact sites A are known to be resistant to EDTA. In NP14, cells began to form EDTA-resistant contacts at about 4hr and most cells at 7 hr remained agglutinated in the presence of EDTA. Pulsed cells became cohesive faster than control cells and maximum agglutination occurred at 5 hr. In

contrast to NP14, HT41 cells scarcely formed EDTA-resistant cell agglutinates. When pulsed with cAMP, however, they were also induced to form such agglutinates (Fig. 5). The induction of contact sites A with cAMP pulses was also observed by Gerisch et al. (1975) and Darmon et al. (1977).

Finally, two enzymes which are known to accumulate at late and post aggregation stage were examined.

Tyrosine transaminase.. In NP14, tyrosine transaminase activity was constant at low level until approxmately 15 hr, and gradually increased thereafter. When cAMP pulses were applied, the enzyme activity began to increase at 9 - 12 hr. Increase in enzyme activity in pulsed cells occurred earlier than that in unpulsed cells. On the other hand, NP14 cells shaken in suspension acquired cohesiveness and formed roughly spherical clumps of a few hundred cells at about 12 - 15 hr, while they constructed the tight clumps at 8 - 11 hr in the presence of cAMP pulses. Therefore, increase in enzyme activity seemed to begin at the time of contact formation among cells. In HT41, enzyme accumulation also occurred slowly after about 15 hr. Cyclic AMP pulses caused precocious accumulation in HT41 as seen in NP14. In this case, change in enzyme activity also coincided with the

time of contact formation (Fig. 6).

Trehalose 6-phosphate synthetase. Accumulation of trehalose 6-phosphate synthetase did not occur up to 24 hr when NP14 cells were shaken in suspension. The enzyme level was unaltered in the presence of cAMP pulses (Fig. 7). However, when cells were developed normally on non-nutrient agar, the enzyme activity began to increase at pseudoplasmodial stage and the increase continued to culmination stage (Fig. 8). In aggregateless HT41, also, no increase in the enzyme activity was observed when cells were shaken in suspension regardless of addition of cAMP pulses (Fig. 7). Even when cells were developed on agar, however, the enzyme activity remained constant at low level (Fig. 8).

As shown, strain HT41 was found to have differences in the accumulation of several enzymes from parental strain NP14, and externally added pulses of cAMP were found to cause various effects on both NP14 and HT41.

DISCUSSION

In D. discoideum, cells developed in suspension can still accumulate some of developmentally regulated enzymes in almost similar manner as they are developed on substratum (Grabel & Loomis, 1977; Rickenberg, Tihon & Güzel, 1978). For accumulation of most enzymes examined here, prior RNA and protein syntheses are required (Roth & Sussman, 1968; Loomis, 1970; Pong & Loomis, 1971).

From the results obtained here, it may be possible to draw some relationship among biochemical events which occur sequentially during development and to deduce factors causing such changes.

Following the initiation of development, activity of β-glucosidase began to decrease and, on the contrary, accumulations of α-mannosidase and N-acetylglucosaminidase occurred (Fig. 1, 2 and 3). Trigger that initiates these changes is starvation. However, starvation signal responsible for accumulation of N-acetylglucosaminidase seems different from that causes the other two responses since only accumulation of N-acetylglucosaminidase among these three responses was not observed in aggregateless strain HT41.

As development proceeds, some cells in population begin to emit cAMP periodically (Robertson et al., 1972;

Gerisch & Hess, 1974). Prior to acquisition of such ability, however, they may need to pass the stage of accumulation of N-acetylglucosaminidase. Because HT41 cells which failed to accumulate this enzyme were also unable to emit cAMP pulses. Accumulation per se may not be necessarily required, however, since mutants which lack this enzyme can still complete the developmental cycle (Dimond, Brenner & Loomis, 1973).

HT41 cells did neither accumulate extracellular phosphodiesterase nor acquire contact sites A but they could do so in the presence of external pulses of cAMP (Fig. 4 and 5). Therefore, periodically emitted cAMP is directly responsible for the initiation of these changes. In NP14, it caused stimulation of accumulation of the enzyme and precocious acquisition of membrane protein (Fig. 5). It is because NP14 cells can emit cAMP pulses even in the absence of external pulses. Similar phenomena were observed by Darmon et al. (1977).

In addition, cAMP pulses stimulated accumulation of α-mannosidase but inhibited accumulation of N-acetylglucosaminidase (Fig. 2 and 3). It is not certain at present, however, whether the decrease of the activity of α-mannosidase after 8 hr development in NP14 is caused directly or secondarily by cAMP pulses. Accumulation of N-acetylglucosaminidase in wild-type strain was completely

inhibited within 2 or 3 hours after commencement of addition of cAMP pulses. This may explain the decrease of enzyme accumulation of unpulsed NP14 cells which was observed at around 9 - 15 hr in development (Fig. 3). Because naturally occurring cAMP pulses begin at about 6 hr after starvation (Robertson et al., 1972).

Initiation of accumulation of tyrosine transaminase occurred at the time of the formation of large cell clumps. Cyclic AMP pulses caused the precocious accumulation of this enzyme (Fig. 6). As formation of cell clumps was also stimulated by cAMP pulses, however, coincidence between these events was also observed. Therefore, cell contacts seem to be responsible for the initiation of accumulation of this enzyme. Grabel & Loomis (1977) also reported the necessity of cell contacts for this change. Contact sites A are probabley involved in this process, because their synthesis is also stimulated by cAMP pulses (Fig. 5).

So long as cells were shaken in suspension irrespective of addition of cAMP pulses, trehalose 6-phosphate synthetase failed to accumulate (Fig. 7). But it occurred at pseudoplasmodial stage when NP14 cells were allowed to develop on agar (Fig. 8). In suspension, large and spherical clumps of cells were the last stage of development. Therefore, cell contacts seem insuffi-

cient for the initiation of accumulation of this enzyme. In addition to them, some special organization of cells or conditions found in slug seem to be required for triggering this change.

Cyclic AMP pulses seem mainly to influence " middle " stage enzymes in development whose activities change during preaggregative and aggregative stage. Accumulations of " early " and " late " enzymes are hardly altered by them. It may be related to the level of cAMP receptors on cell surface because cells at early and late stage of development can not bind sufficient amount of cAMP (Malchow & Gerisch, 1974).

Externally applied pulses of cAMP caused various effects on the enzyme accumulation, such as inhibition, stimulation or induction. It is interesting to know how these diverse reponses are caused by a single species of molecule. It may be essentially the same problem as the hormonal control of the metabolism in higher animals.

SUMMARY

In wild-type strain NP14 and signal- and relay-less mutant HT41, changes of activities of six developmentally regulated enzymes and of one membrane protein during development were examined. Effects of externally added cAMP pulses on them were also examined.

HT41 differed from NP14 in accumulation of both N-acetylglucosaminidase and extracellular phosphodiesterase and acquisition of contact sites A. However, cAMP pulses induced the latter two events in HT41. They did not alter the disappearance of β-glucosidase activity but stimulated accumulation of α-mannosidase. Accumulation of N-acetylglucosaminidase in NP14 was inhibited by them. They also caused precocious accumulation of tyrosine transaminase. Trehalose 6-phosphate synthetase failed to accumulate in suspension irrespective of them, but occurred at pseudoplasmodial stage during development on agar. The causal relationship between these biochemical events and factors responsible for these changes are discussed.

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Fig. 1. Changes of β-glucosidase activity and effect of cAMP pulses during the development of wild-type NP14 and aggregateless HT41. Cells were shaken in suspension with or without the addition of cAMP pulses. At intervals, β-glucosidase activity was assayed. Pulse treatment was began at the time indicated by arrow. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (Δ), HT41 without pulses; (▲), HT41 with pulses.

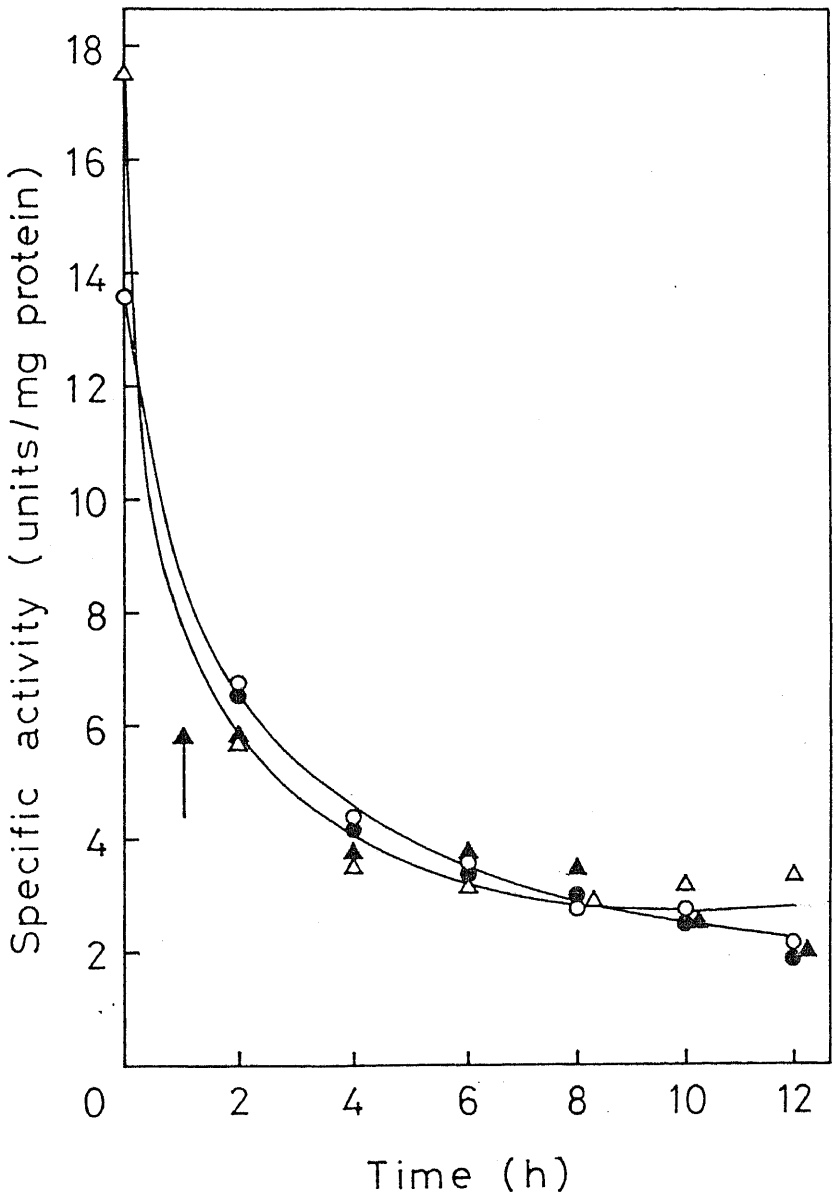

Specific activity (units/mg protein)
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
Time (h)

Fig. 2. Changes of α-mannosidase activity and effect of cAMP pulses. Enzyme activity was assayed as descibed in the legend of Fig. 1. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (Δ), HT41 without pulses; (▲), HT41 with pulses.

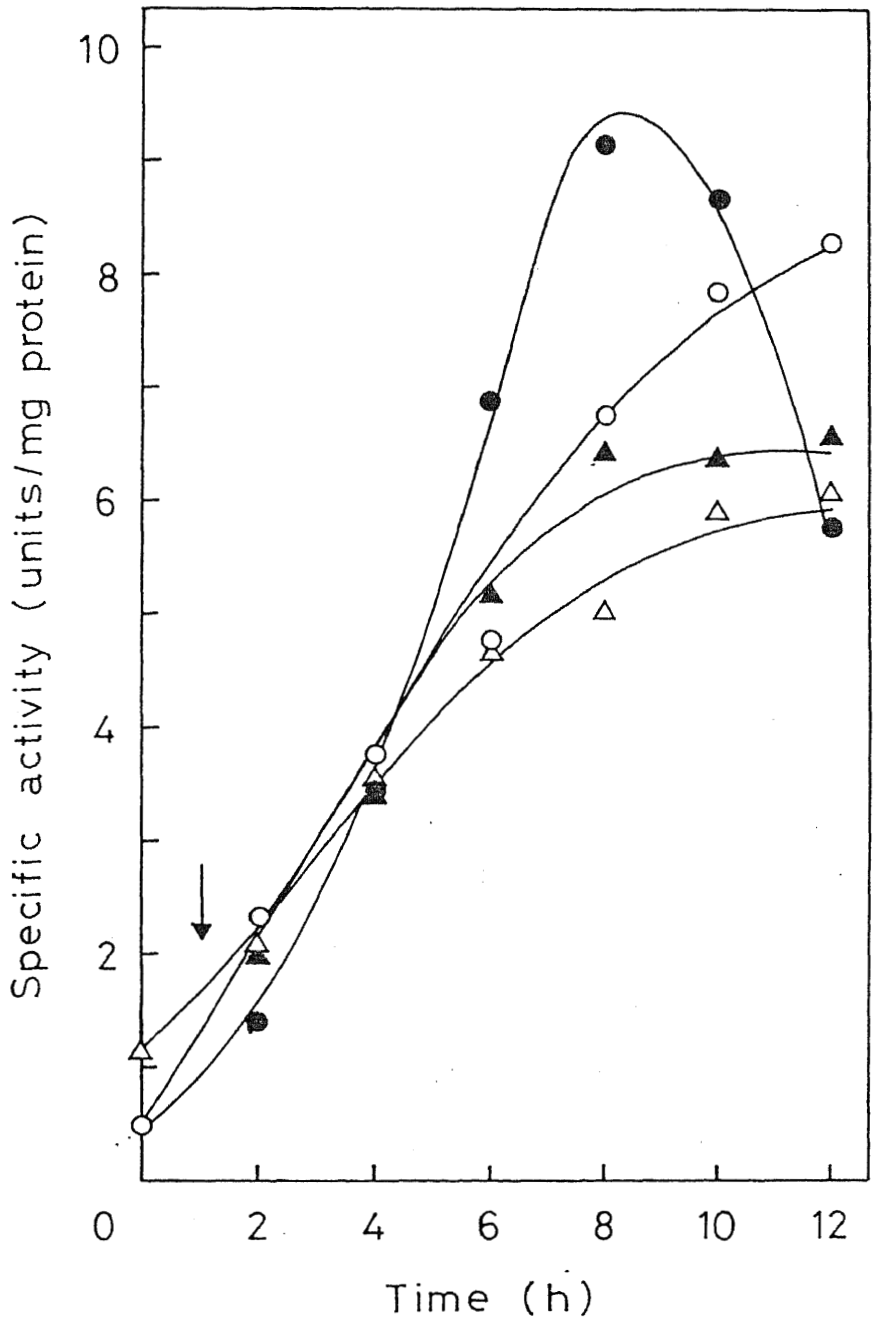

Specific activity (units/mg protein)
10
8
6
4
2
0
2
4
6
8
10
12
Time (h)

Fig. 3. Changes of N-acetylglucosaminidase activity and effect of cAMP pulses. Enzyme activity was assayed as described in the legend of Fig. 1. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (△), HT41 without pulses; (▲), HT41 with pulses.

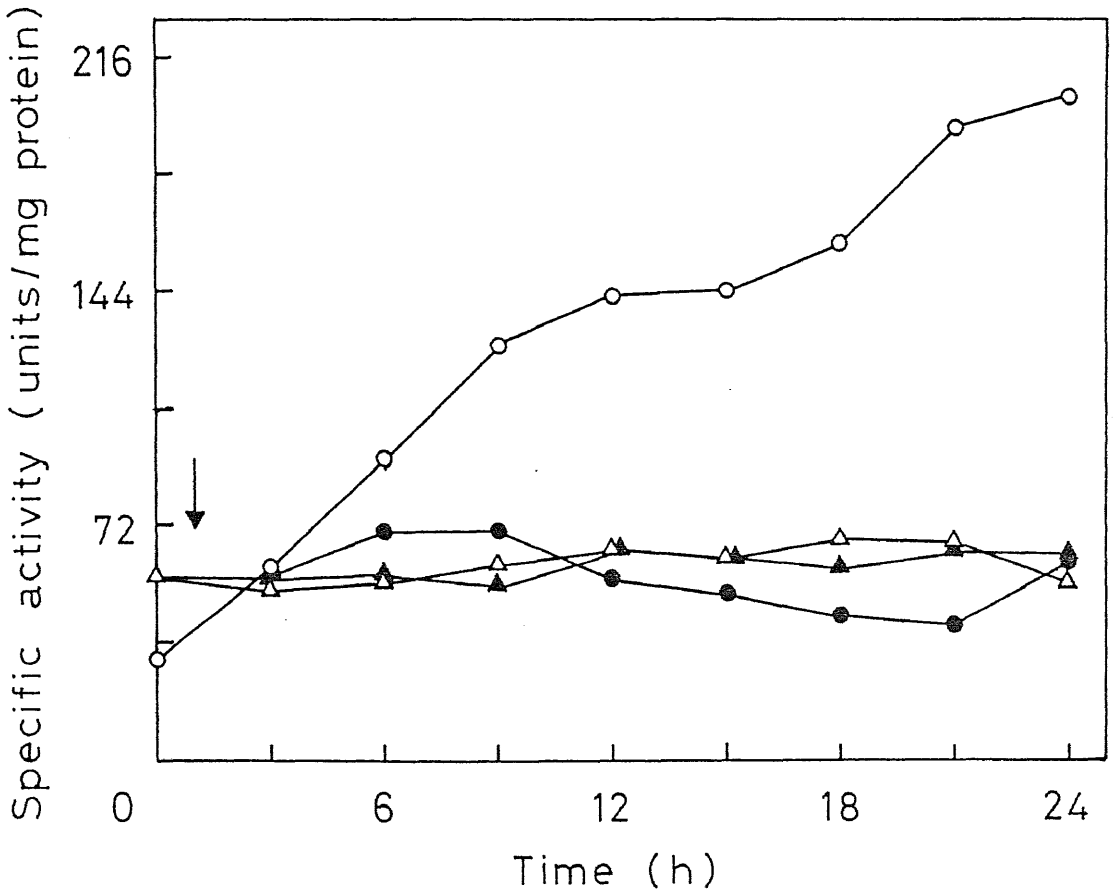

Specific activity (units/mg protein)
216
144
72
0
6
12
18
24
Time (h)

Fig. 4. Changes of extracellular phosphodiesterase activity and effect of cAMP pulses. Enzyme activity was assayed as described in the legend of Fig. 1. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (Δ), HT41 without pulses; (▲), HT41 with pulses.

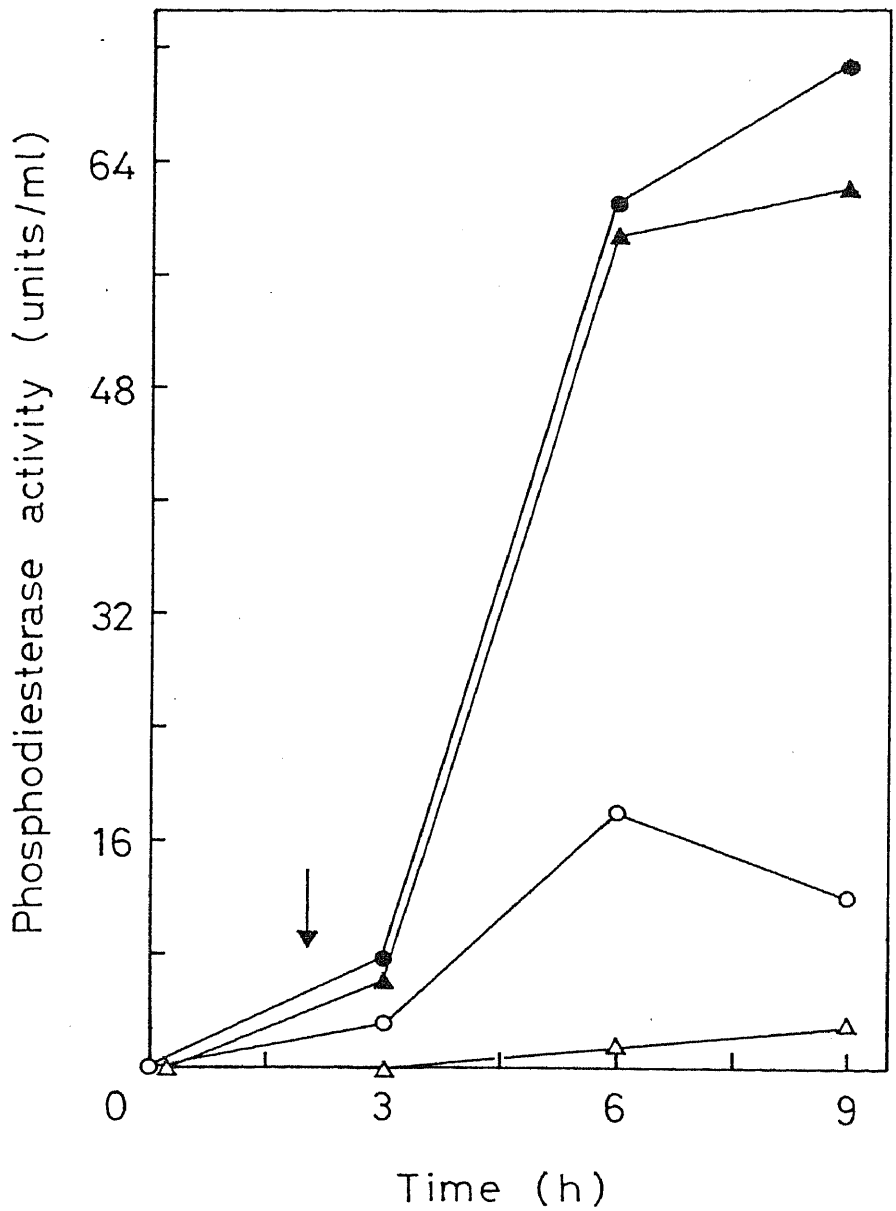

Phosphodiesterase activity (units/ml)
64
48
32
16
0
3
6
9
Time (h)

Fig. 5. Acquisition of contact sites A and effect of cAMP pulses. Cells were shaken in suspension with or without the addition of cAMP pulses. At intervals, cells dissociated in EDTA solution were counted. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (Δ), HT41 without pulses; (▲), HT41 with pulses.

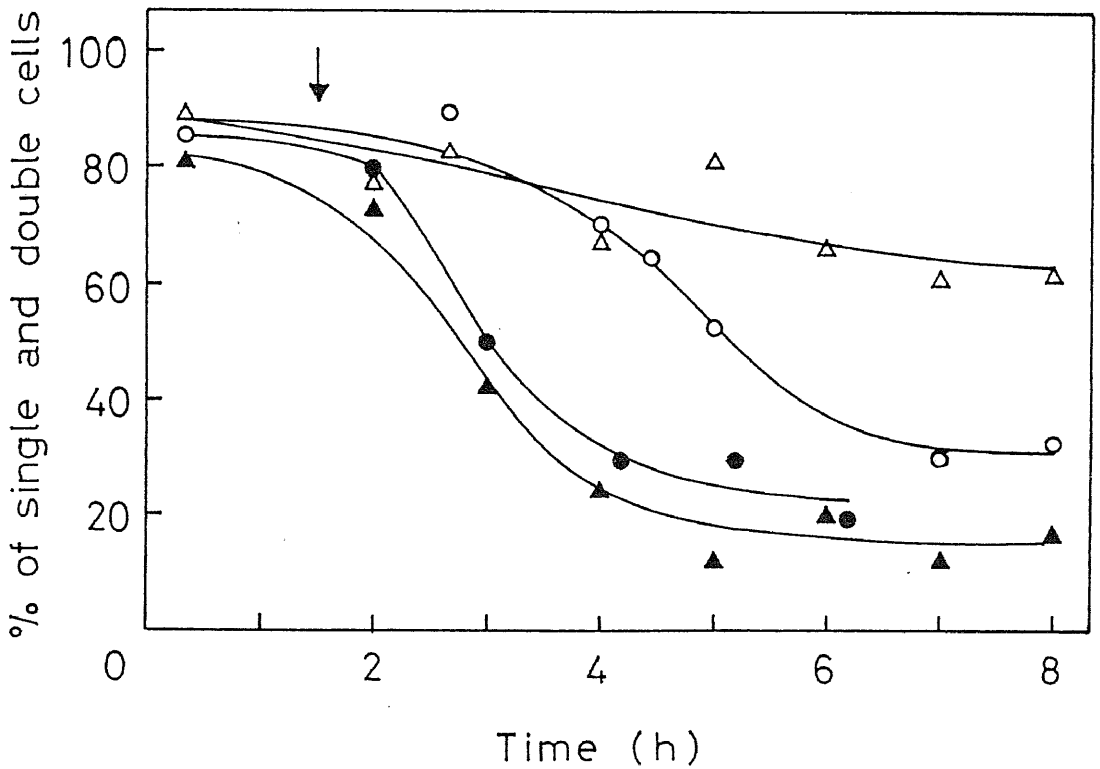

% of single and double cells
100
80
60
40
20
0
2
4
6
8
Time (h)

Fig. 6. Changes of tyrosine transaminase activity and effect of cAMP pulses. Enzyme activity was assayed as described in the legend of Fig. 1. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (Δ), HT41 without pulses; (▲), HT41 with pulses.

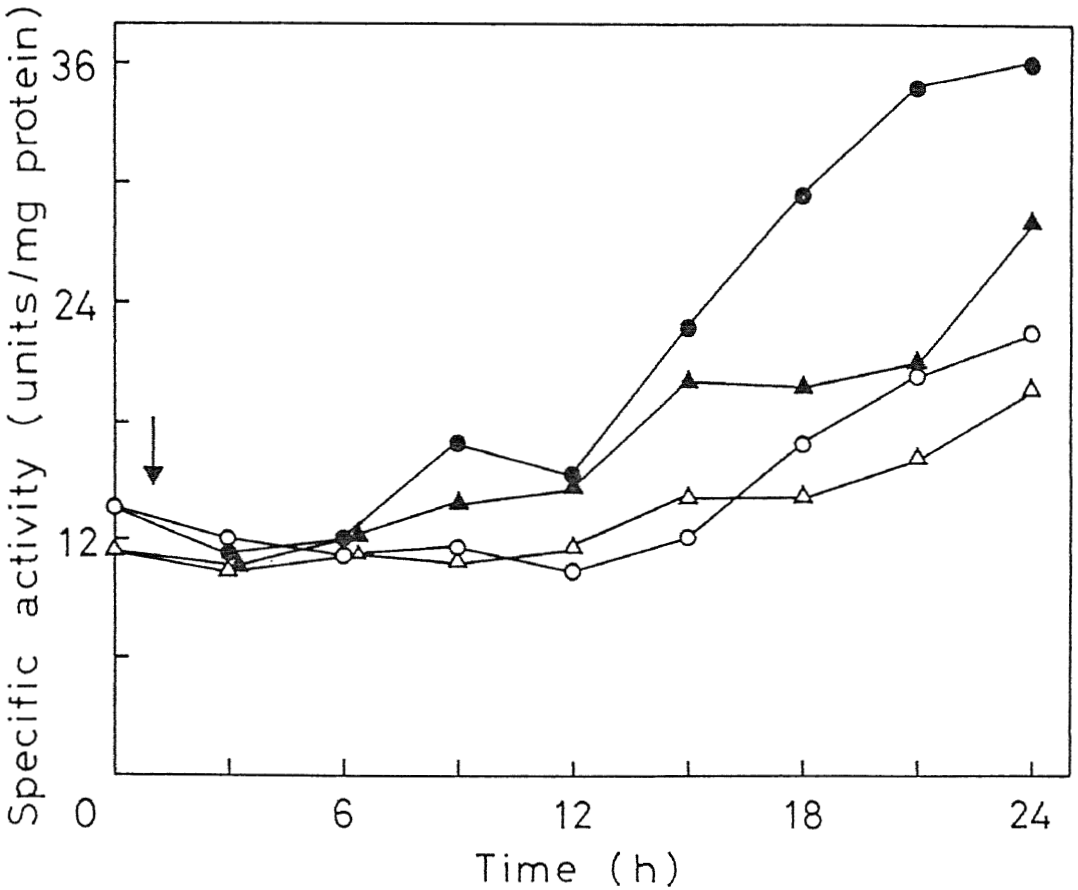

Specific activity (units/mg protein)
36
24
12
0
6
12
18
24
Time (h)

Fig. 7. Changes of trehalose 6-phosphate synthetase and effect of cAMP pulses. Enzyme activity was assayed as described in the legend of Fig. 1. (o), NP14 without cAMP pulses; (●), NP14 with pulses; (△), HT41 without pulses; (▲), HT41 with pulses.

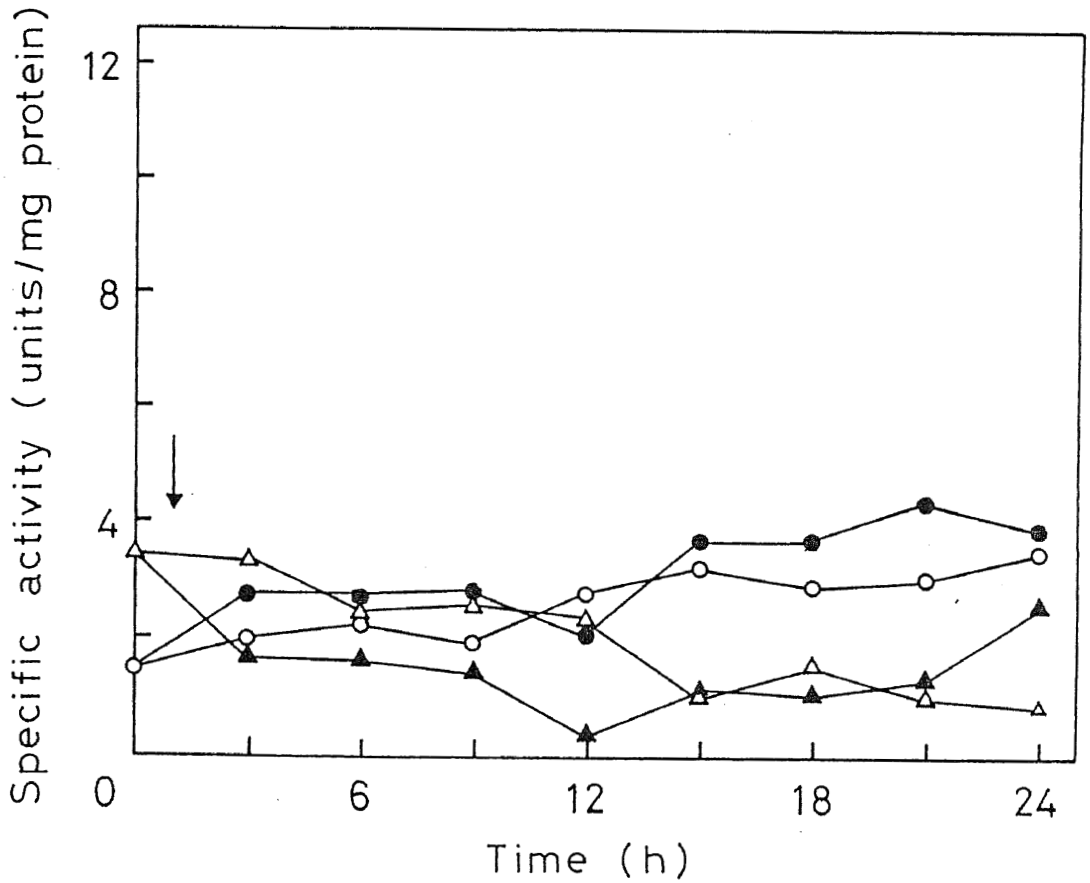
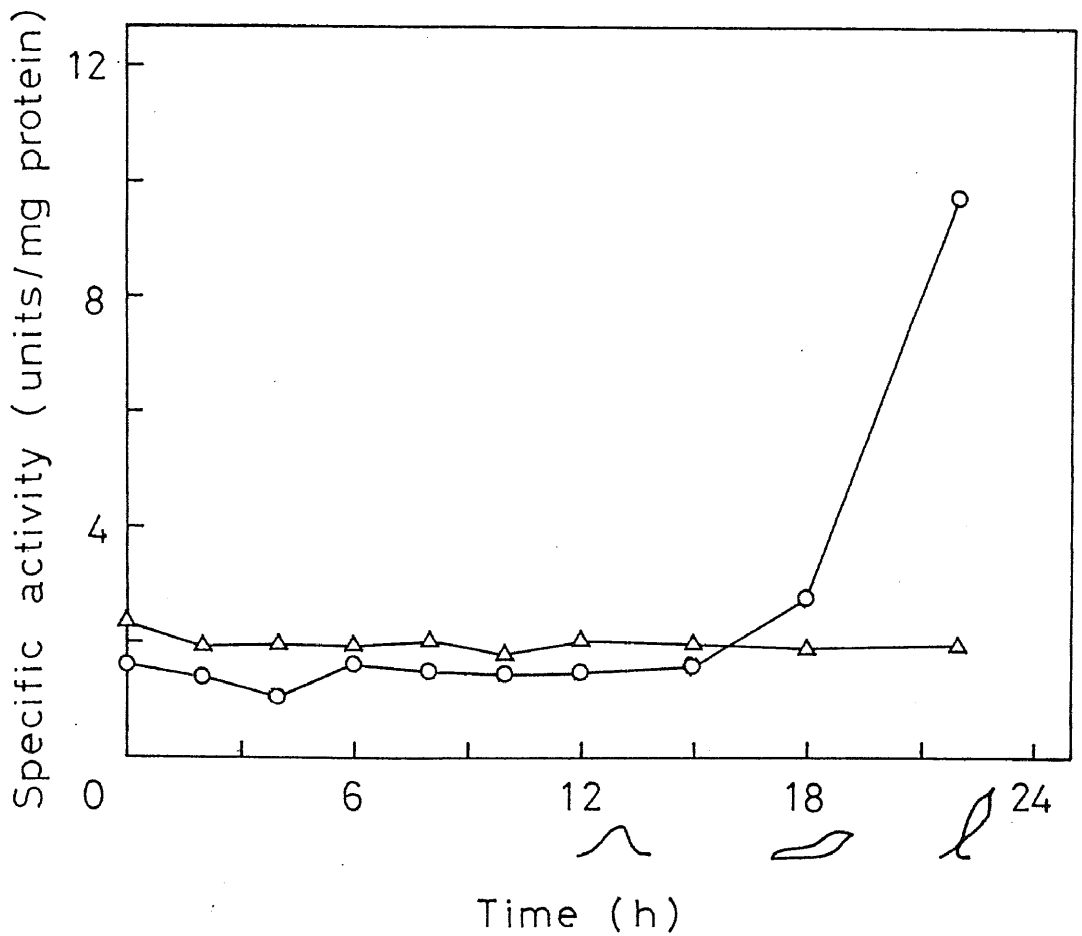

Specific activity (units/mg protein)
12
8
4
0
6
12
18
24
Time (h)

Fig. 8. Changes of trehalose 6-phosphate synthetase activity. Cells were allowed to develop on non-nutrient agar. At intevals, cells were harvested and enzyme activity was assayed. (o) NP14 and (Δ) HT41.


Specific activity (units/mg protein)
12
8
4
0
6
12
18
24
Time (h)

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