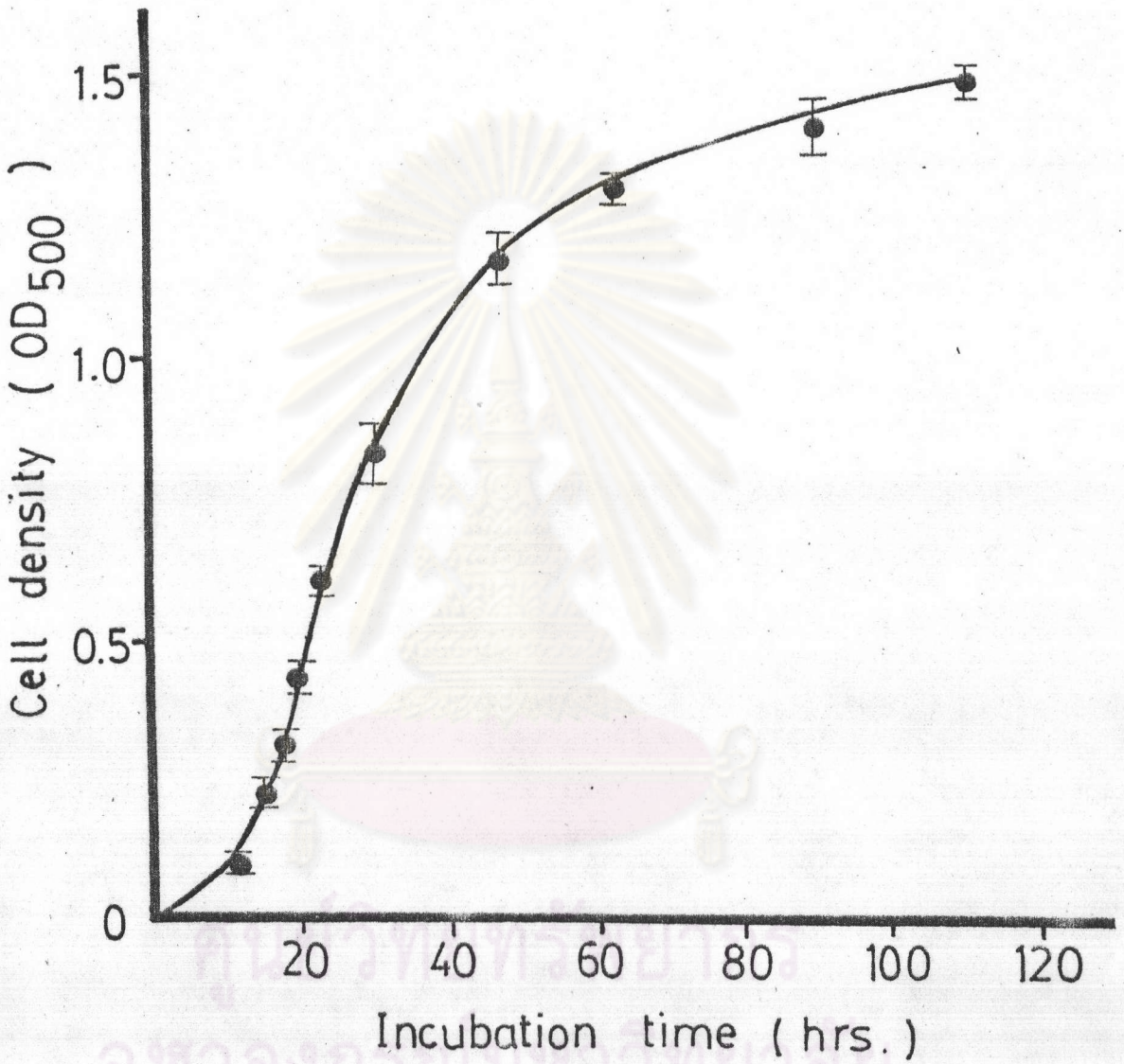
CHAPTER III

RESULTS

1. Growth characteristics of R. phaseoli.

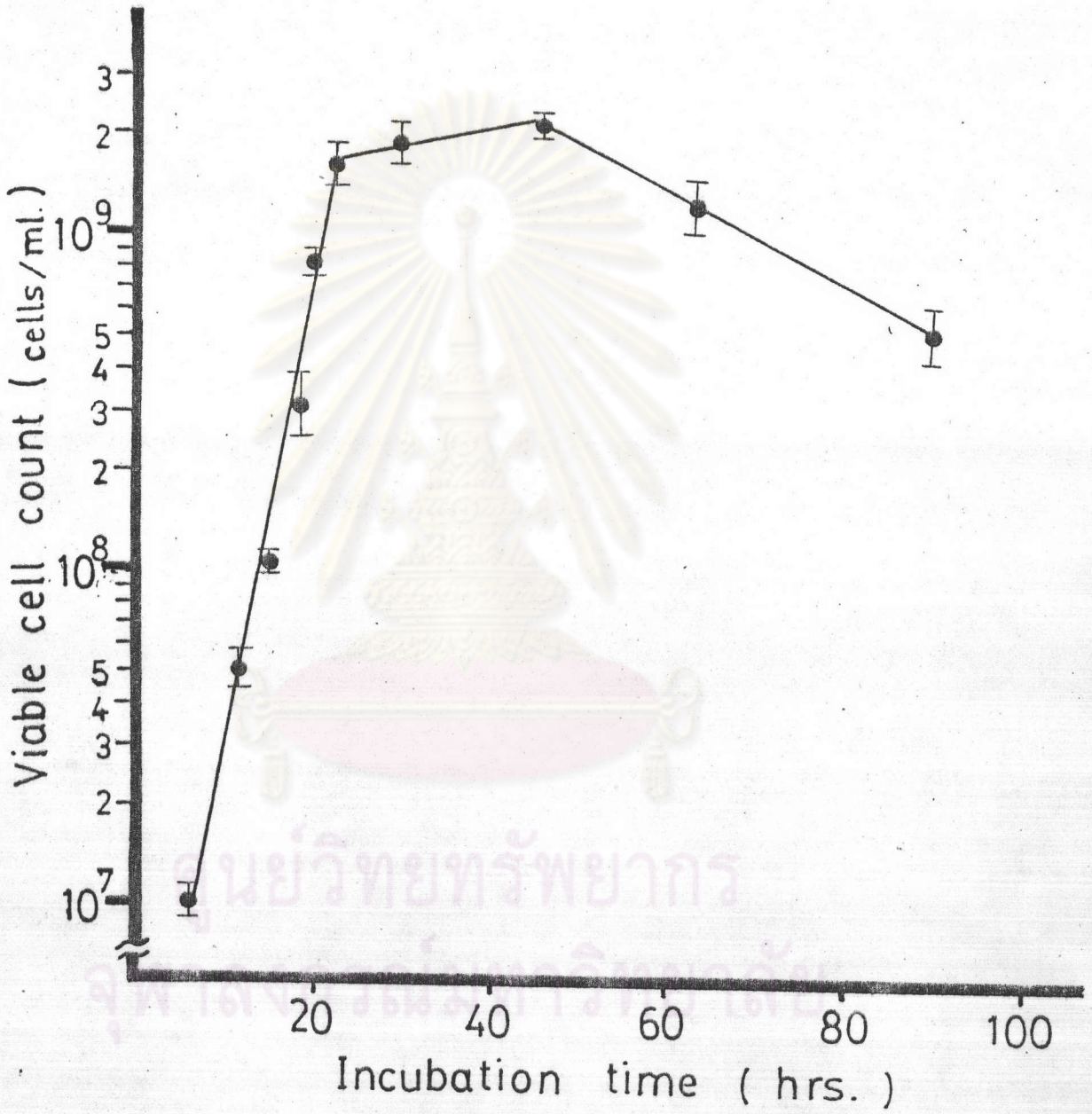
Monitoring of growth of Rhizobium by a direct measurement of an optical density at 500 nm may produce an uncorrect result, since the excreted slime produced by Rhizobium might be the cause of deviation. To verify that an OD or KU. reading could represent growth of bacterium, a viable cell count was followed in accordance with a cell turbidity during the whole growth period. Plot between cell density and viable cell count during the 5 days period of growth were shown in Figure 1 and 2 respectively. Replot between viable cell count versus the repective OD_{500} and KU were shown in Figure 3. These curves were our standard curves used to convert a unit of cell density in terms of OD_{500} or KU into viable cell count. Whenever, a generation time or doubling time of cell growth was required, a conversion of the recorded OD or KU to the corresponding viable cell count was employed by these three standard curves. Limitation of the conversion was restricted only in the linearity of the curve which was around mid-log phase of cell growth. For example, an estimation of the generation time of growth shown in Figure 1 was performed from the reading of 0.4 unit of OD_{500}. At this point, the viable cell count was equal to 4×10^8 cells per ml. By shifting the estimation into Figure 2, the value of 4×10^8 was correlated to 18 hrs and the value of 2×10^8 was 16 hrs, the generation time was, therefore, equal to 2 hrs.

Figure 1. Relative of cell density with respected to incubation time



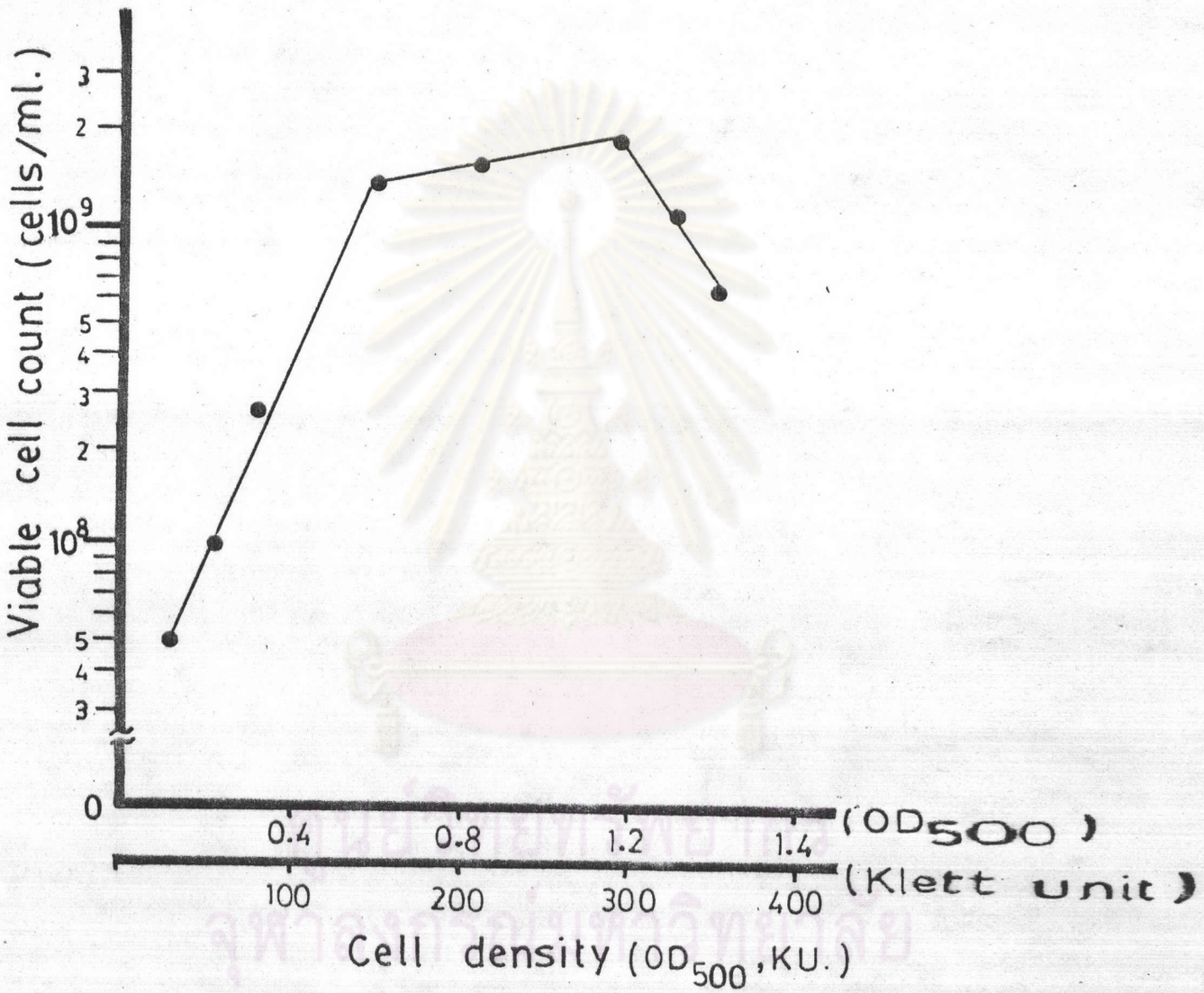
The growth pattern of R. phaseoli TAL 113 (WT) was obtained by a measuring of cell density at each specific time of incubation.

Figure 2. Relation of viable cell count with respected to incubation time



The growth pattern of R. phaseoli in YM was obtained by a measuring of viable cell count at each specific time of incubation.

Figure3. A correlation of cell density and viable cell count.



The cell density was plotted versus the corresponding viable cell count at each specific point of incubation time.

2. Selection of salt-tolerant mutants.

The aim of our project was to select salt-tolerant strains by an application of a sibling selection. Initially, 50 samples of an individual colony were grown in YM media containing NaCl at a concentration ranging from 0.01-0.3 M. Bacterial cultures containing salt lower than 0.1 M were turbid after a few days of incubation. Cell cultures from 0.1 M NaCl solution were regrown on 0.1 M NaCl YM congo red plate. Ten isolates, picked up from the second grown 0.1 M NaCl YM plates, were pooled in the same flask. Ten flasks, containing YM medium with NaCl at a concentration ranging from 0.1-0.5 M. were then prepared. Only one from the ten samples could regrown on YM medium containing NaCl at a final concentration of 0.3 M after a 5 day incubation. Cell were regrown on YM plate plus 0.3 M NaCl. Twenty two strains were randomly picked up and purified and then subjected to test for nodulation ability. It was found that, four out of 22 strains tested possessed the nodulation ability and were named as P_1, P_5, P_{19} and P_{21} which was used throughout this study.

3. Symbiotic ability of the isolated mutants : A test of.

Nodulate ability of the salt-tolerant strains isolated was the most crucial property of our goal. We, therefore, performed a Leonard's test as described in the Methods section 4. Plants of one month old, planted under described condition, being formerly inoculated with WT and salt-tolerant strains were our samples. Plants, free from any inoculation were used as our control. Roots cut from planta with and without inoculation were photographed together as shown in Fig 4a. Roots cut from planta being inoculated with salt tolerant strains; P_1, P_5, P_{19} and P_{21} were separately shown in Fig 4b, 4c, 4d and 4e respectively. At an instant observation, roots obtained from plants being nodulated by salt-tolerant strains were glimpsed a variation in size. However, all of them provided the similar healthy character as

the same as that from WT. All plant tops were used to determine plant wet weight, plant height, nodules number and root activity of acetylene reduction (ARA.). The whole data were analysed statistically. The details of the statistical analysis were shown in Appendix 1-4. Only the final numberical values from the statistical analysis were summarized in Table 2. Summation derived from the statistical treatment was as follows. First, when nodule number was regarded as a parameter and the data were subjected to Duncan's new multiple range test, it was found that plant of different nodule numbers but of the same mark (letter a, b or c) provided a non-significant difference at 0.05% confidence level. Second, the rest of other parameters ie., ARA.; plant wet weight and plant height were led to the same conclusion that there was no significant difference at 0.05% confidence level regardless of having a group variation in nodule numbers which have been remarked in a, b or c. It was worthwide to note that ranges of % coefficiency of variation (%CV.) found, could be devided into two groups. Nodule numbers and ARA. were falled in the same group whereas the plant height and plant wet weight were the orthers.

Figure. 4 Nodule formation of plant inoculated with WT and its mutants.

One month old plants inoculated with WT and its mutants were drawn from Leonard's jar. The roots were washed and observed for nodule formation.

(a) uninoculated and inoculated with WT.

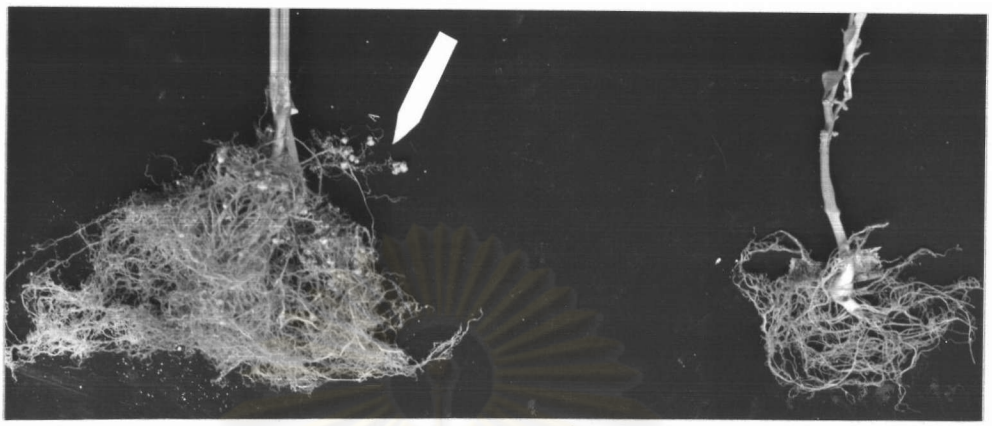
(b) inoculated with the mutant P_1

(c) inoculated with the mutant P_5

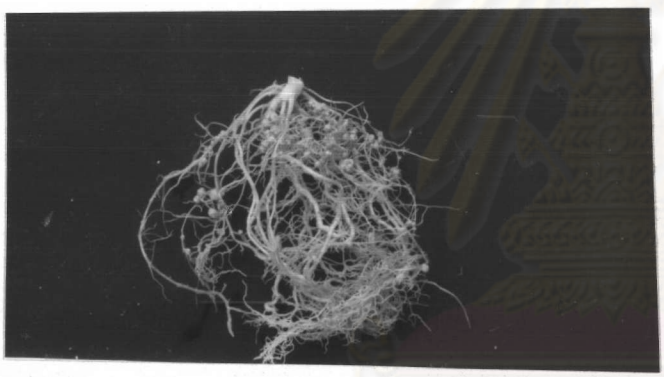
(d) inoculated with the mutant P_{19}

(e) inoculated with the mutant P_{21}

(a)



(b)



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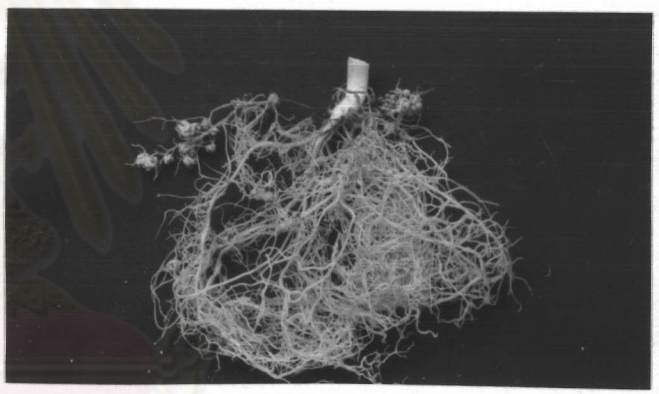
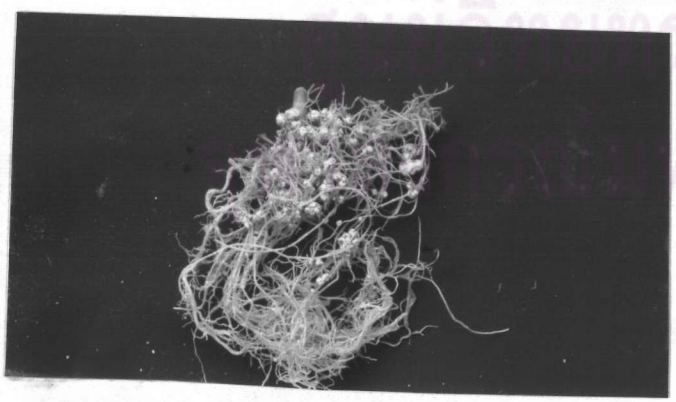


(d)



(e)

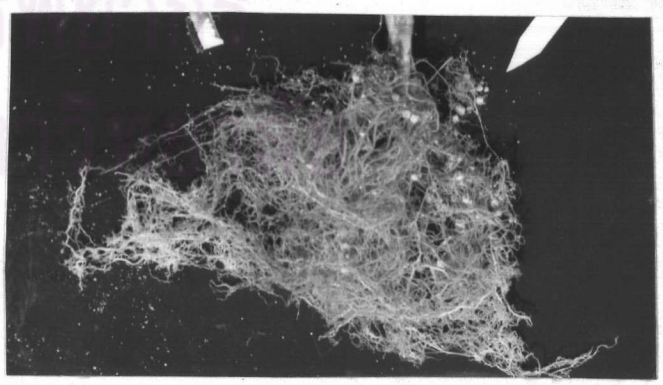


Table 1. Parameter of growth from one month old planta which being nodulated by WT and its mutants.

Strain	No. of nodules per plant	Dry wt. of nodule per plant (mg)	Wet wt. of nodule per plant (mg)	A.R.A (µmole/hr/g nodule)	Plant height (cm)	Wet wt. per plant (g)
WT	12	15	150	31.3	-	2.3
	90	54	300	13.0	-	3.6
	15	5	20	10.0	30	3.4
	60	21	110	12.0	33	4.0
	40	46	350	6.1	35	5.8
	35	29	230	35.5	38	4.6
	166	80	270	17.5	39	6.1
	171	110	400	13.0	33	4.8
P_1	70	130	520	7.4	-	4.4
	60	60	320	1.2	39	5.1
	26	7	400	8.6	37	4.6
	75	40	270	10.3	37	4.6
	7	10	640	5.6	38	6.2
P_{19}	100	120	440	17.5	-	3.0
	230	180	750	8.2	40	5.8
	190	100	450	13.4	40	5.0
	80	10	110	16.4	39	6.2
	47	7	50	28.6	35	4.2
	47	8	50	20.0	38	5.8
P_5	40	20	120	8.5	-	5.0
	20	4	16	12.5	42	6.2
	20	3	14	13.3	30	4.8
	25	4	40	25.0	42	5.5
	20	9	80	16.7	30	4.4
	7	36	65	17.0	40	5.0
P_{21}	6	14	110	5.0	-	3.1
	20	7	36	21.4	32	4.0
	10	3	30	13.3	35	3.1
	15	4	40	12.5	35	3.9
	8	5	48	80.0	44	6.1
	15	14	44	33.0	40	7.0
	3	6	28	5.0	42	6.2
Control	-	-	-	-	40	3.2
	-	-	-	-	37	2.9
	-	-	-	-	30	3.3
	-	-	-	-	37	3.9
	-	-	-	-	30	3.0
	-	-	-	-	30	4.4

Table. 2 The final numerical values of plant samples after treated with statistics

(1) By the application of Duncan's new multiple range test, group of plant samples which having nodule numbers of no significant difference were earmarked by the same letter (a, b or c).

(2) ARA, plant wet weight and plant height were subjected to F' test all of the last numerical values obtained, indicated no significant difference among group of planta being test of.

Table 2. Statistical analysis of the symbiotic properties, among group of plant samples being inoculated with WT or its mutants.

Strain no.	No. of nodules per plant (1)	Acetylene reduction Activity (ηmoles C_2H_2/hr/g nodule)$_2$	Wet weight per plant (2)$_2$	Height (cm)$_2$
WT	73 ab	17.3	4.32	34.7
P_1	48 bc	6.6	4.98	37.8
P_5	22 bc	15.5	5.15	36.8
P_{19}	115 a	17.4	5.00	38.4
P_{21}	11 c	24.3	4.77	37.7
Control	-	-	3.45	34.0
% C.V.[3]	37.8	39.0	13.8	4.9

4. Growth of salt-tolerant mutants.

Photographs in Fig. 5 showed the ability to form colony among mutants in the presence of 0.3 M NaCl. Colonial size of tested mutants was similar whereas no growth was observed for WT. All mutants and WT were reused to observe growth in liquid culture with and without 0.3 M NaCl and results were illustrated in Fig. 6. Viewed from the growth profiles, the ability to multiply in YM medium without salt of both WT and all mutants was relatively the same, except a little lower in value of OD_{500} at the stationary phase was observed in mutants. However, when cultivation was performed in the presence of 0.3 M NaCl, no growth was observed in WT, whereas normal growth was found in mutants. Points to be noted were that, a doubling time of mutants in the presence of 0.3 M NaCl was markedly increased 4-6 times as compared to that of without salt, and a rather low value in cell density at stationary phase. Summation of the significant growth values of WT and mutants was shown in Table 3.

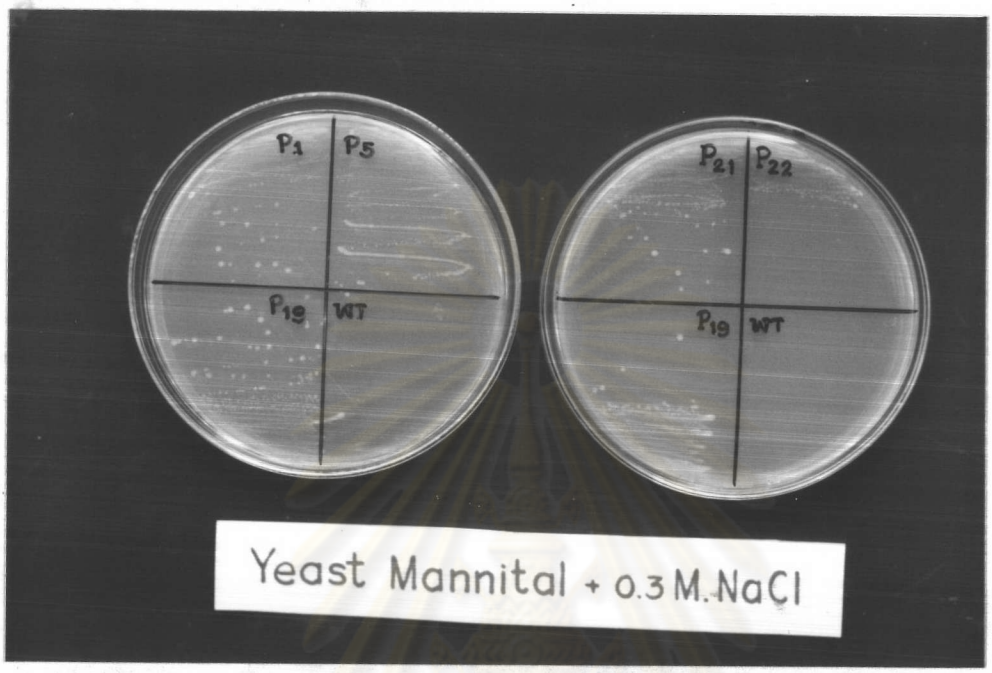


Figure 5 Colonial morphology of the isolated salt-tolerant mutants on YM + 0.3 M NaCl plate.

The isolated colonies of wild type and its mutants were streaked on YM + 0.3 M NaCl. and incubated at 30°C for 7 days.

WT = wild type

P_1, P_5, P_{19}, P_{21}, P_{22} = salt-tolerant mutants

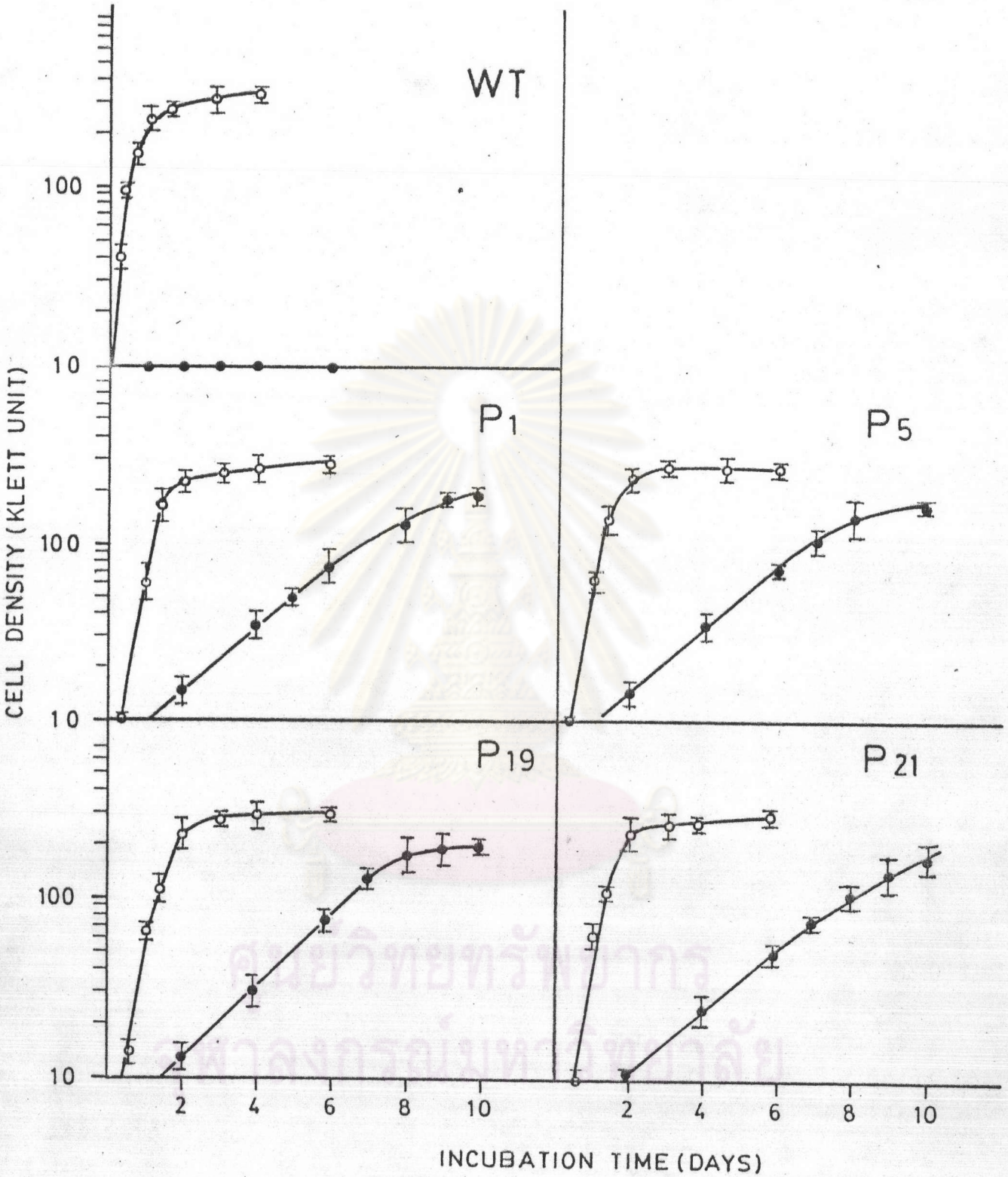


Figure 6. Comparison of growth profiles between WT and its mutants.

o———o grown in YM

●———● grown in YM + 0.3 M NaCl

WT = Wild type

P_1, P_5, P_{19}, P_{21} = mutant strains

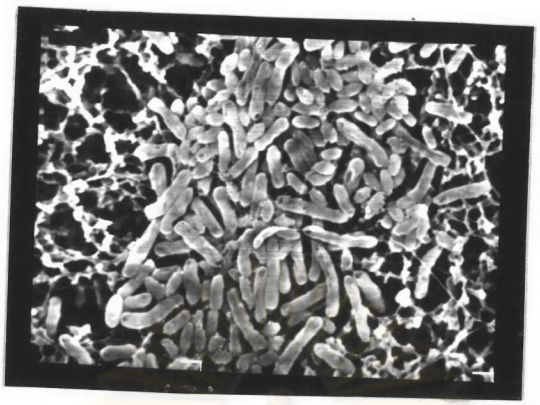
Table 3. Character of growth of WT and its mutants under salted and unsalted condition.

strain	log phase[1] duration (d)	generation time[1] (hr)	growth yield[1] (Klett-unit)
WT in YM	0.5	2	350
WT in YM + 0.3 M NaCl	-	-	-
P_1 in YM	1.1	4.5	290
P_1 in YM + 0.3 M NaCl	5.0	24.0	200
P_5 in YM	1.4	4.5	259
P_5 in YM + 0.3 M NaCl	5.0	19.0	185
P_{19} in YM	1.2	4.0	300
P_{19} in YM + 0.3 M NaCl	5.0	19.0	210
P_{21} in YM	1.3	4.0	275
P_{21} in YM + 0.3 M NaCl	5.0	24.0	185

[1]All values were the summation taken from Fig. 6.

5. The studies of cellular morphology by SEM and TEM

Vreeland et al. (1984) was the first who reported that, in Halomonas elongata, there was a difference in the cellular morphology in response to increment of salt concentration in its growth medium. We persued this idea by an inspecting of the alteration in the cellular morphology based on the SEM and TEM, using cells which were cultivated with and without 0.3 M NaCl as samples. In case of SEM, mutant strain P_{19} was used as the sole representative SEM pictures performed under our condition, as shown in Fig. 7a, b, c; illustrated no significant difference in the cellular morphology between the WT and mutants, regardless of the cultivation condition. In case of TEM, mutants no. P_5, P_{19}, P_{21} as well as WT were used as representatives. There were some differences in cellular morphology among WT and its mutants. First, there was a surface irregularity around outer phase of the cell envelope of P_5, P_{19} and P_{21} when culturing in the presense of salt. Less surface irregularity was observed when they were cultured in the absence of 0.3 M NaCl. Second, a cytoplasmic compact was observed as was viewed from the denser granule of cytoplasma protion of cell cultivated under 0.3 M NaCl. (Fig. 8-11).

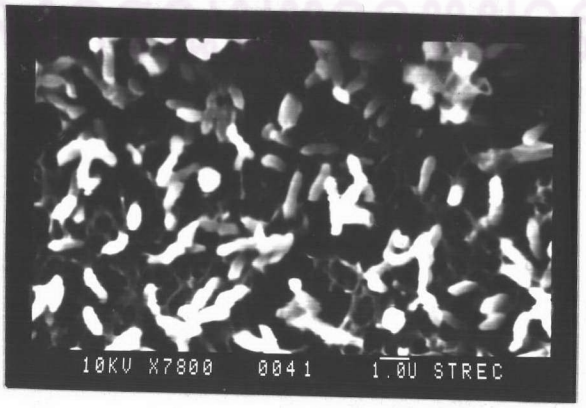


(a)

x 7800 , — (1.0 micron)

(b)

x 7800 , — (1.0 micron)



(c)

x 7800 , — (1.0 micron)

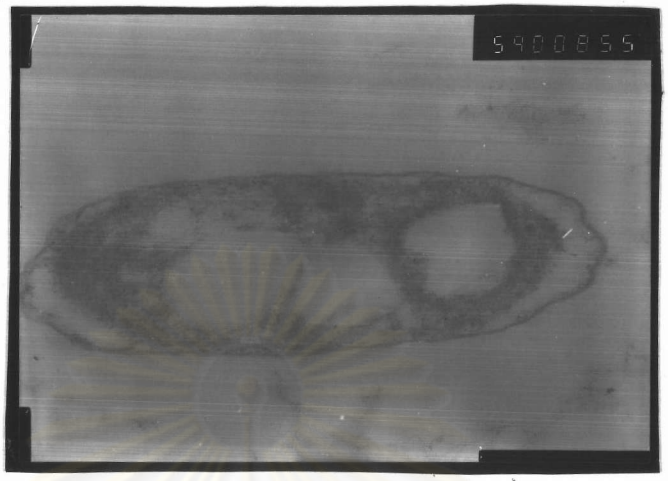
Fig. 7. Scanning electron micrographs of WT and its mutant grown in YM and YM + 0.3 M NaCl.

Cultures of WT and mutant P_{19} grown either in YM or YM + 0.3 M NaCl were prepared for scanning electron microscope as previously described in the Appendix 7,

(a) WT grown in YM

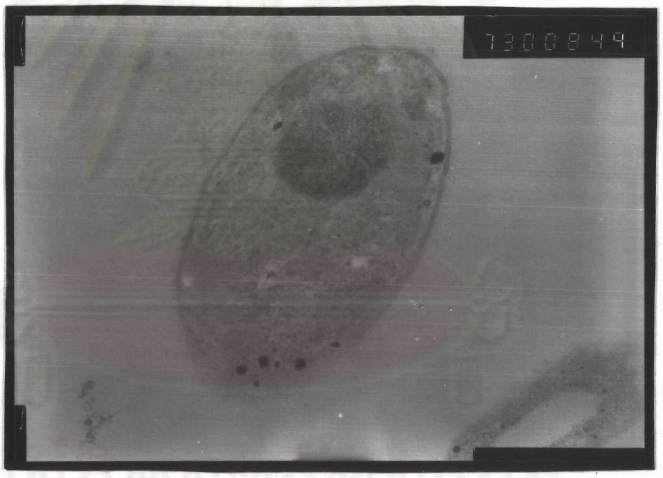
(b) P_{19} grown in YM

(c) P_{19} grown in YM + 0.3 M NaCl.



(a)

X 59,000



(b)

X 73,000

Figure 8. Transmission electron micrograph of WT

Cells grown in YM, were prepared for transmission electron microsope as described as Methods section.

a) Longitudinal section of WT

b) Transversional sector of WT

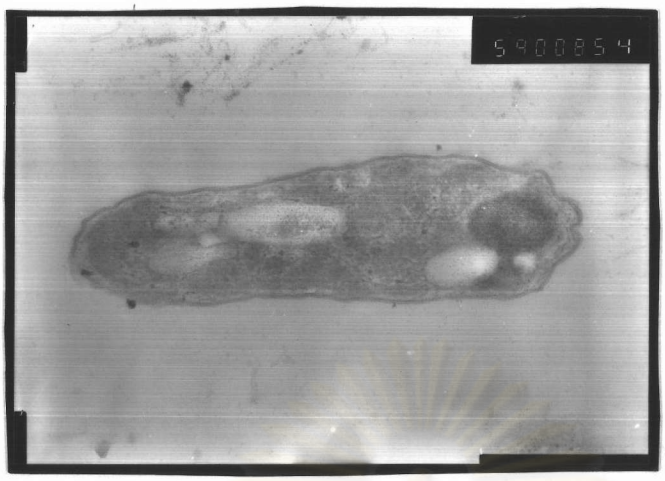
Figure 9. Transmission electron micrographs of mutant P_5 grown under salted and unsalted condition.

Fig. 9a. Thin section of mutant P_5 grown in YM.

Fig. 9b. Thin section of mutant P_5 grown in YM + 0.3 M NaCl

Fig. 9c. Transversional section of mutant P_5 grown in YM + 0.3 M NaCl

9a.

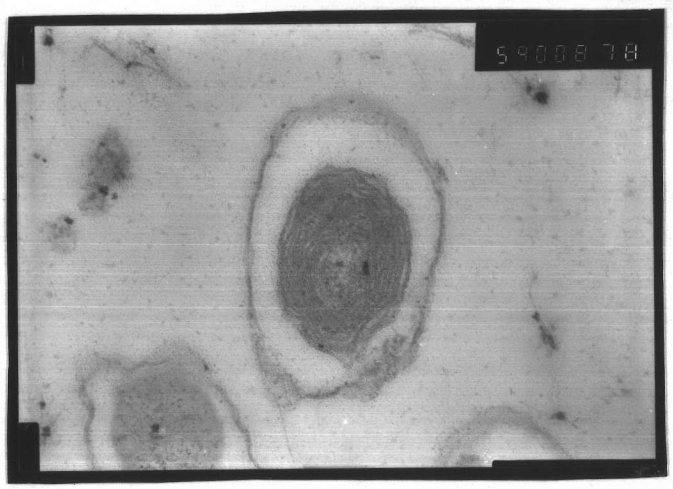


x 59,000

9b.

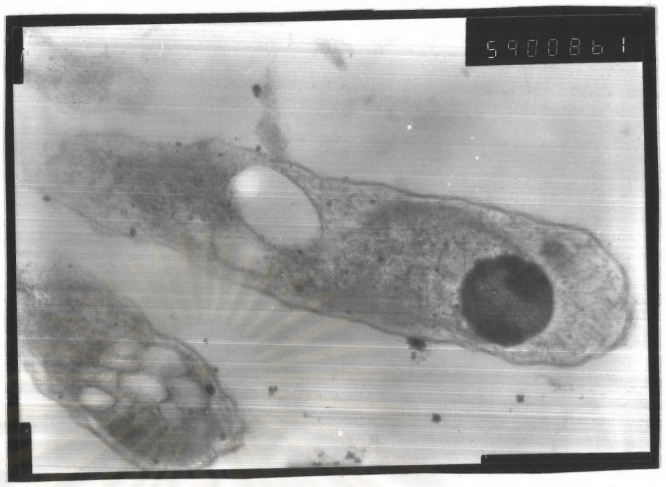
x 30,000

9c.



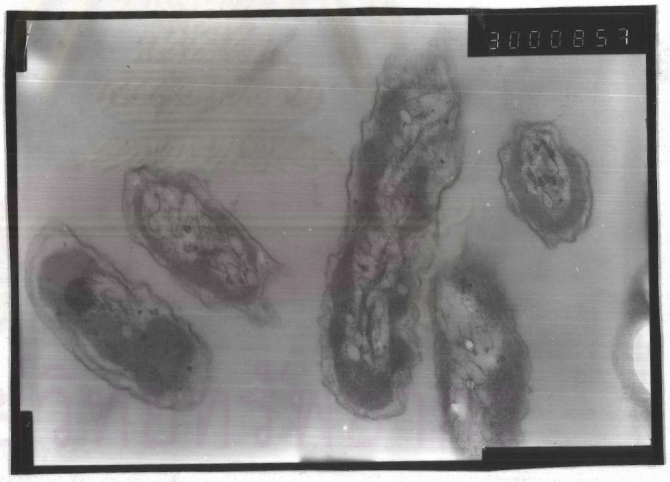
x 59,000

10a.



× 59,000

10 b.

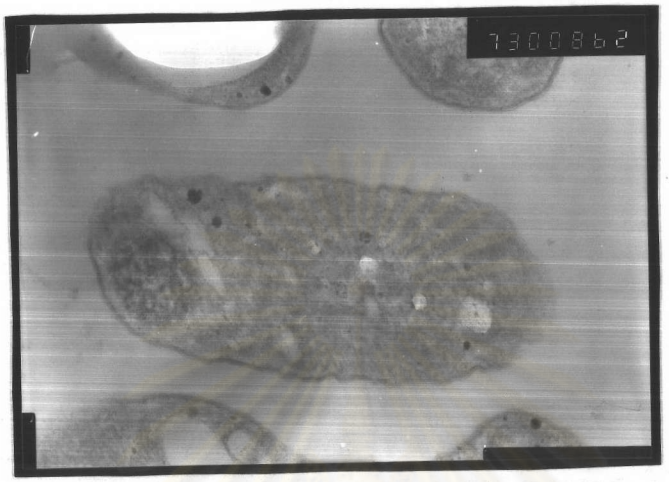


× 30,000

Figure 10. Transmission electron micrograph of mutant P_{19} grown under salted and unsalded condition.

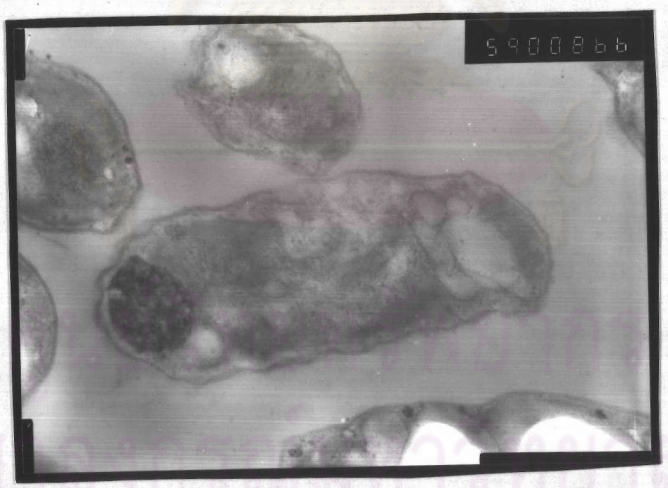
Fig. 10a. Mutant P_{19} grown in YM

Fig. 10b. Mutant P_{19} grown in YM + 0.3 M NaCl.



(a)

× 73,000



(b)

× 59,000

Figure 11. Transmission electron micrographs of mutant P_{21} grown under salted and unsalted condition.

Fig. 11a. Mutant P_{21} grown in YM

11b. Mutant P_{21} grown in YM + 0.3 M NaCl.

6. Checking of the difference on cell surface antigen by fluorescent antibody technique.

Owing to the presence of growth retardation found among mutants being cultivated under salted condition, it was therefore, essential to test whether their cell surface antigen were the same as that of the WT. In order to assure that they all were derivatives of the same strain. Thus, a checking of cell surface antigen based on the fluorescent antibody technique as described in the Methods section 6 was performed. Fig. 12 was the fluorescent micrograph of WT stained with the fluorescent antibody and Fig. 13 were those of mutants stained with the same fluorescent antibody. All of the stained cells found in both figures were identical but obviously different from those of the R. japonicum, the slow growing rhizobium (Fig. 14), which was used as the positive control.

Figure 12. Fluorescent micrographs of R. phaseoli (WT.)

Culture grown in YM was performed. The procedure of checking the cell surface antigen by fluorescent antibody was described in the Methods section 6.

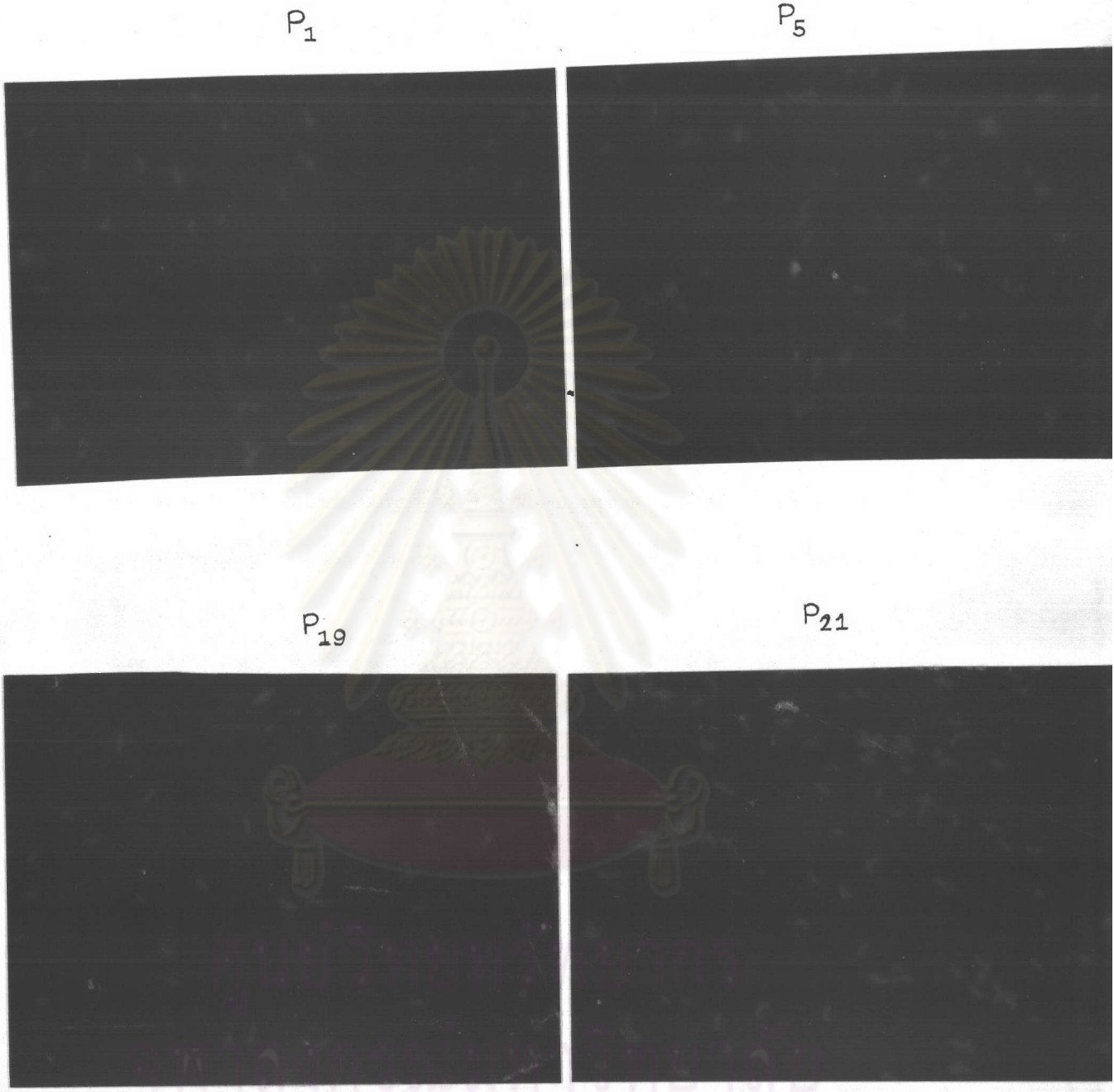


Figure 13. Fluorescent micrographs of the salt-tolerant-mutants.

All mutants grown in YM + 0.3 M NaCl were checked for cell surface antigen by fluorescent antibody technique as described in the Methods section 6.

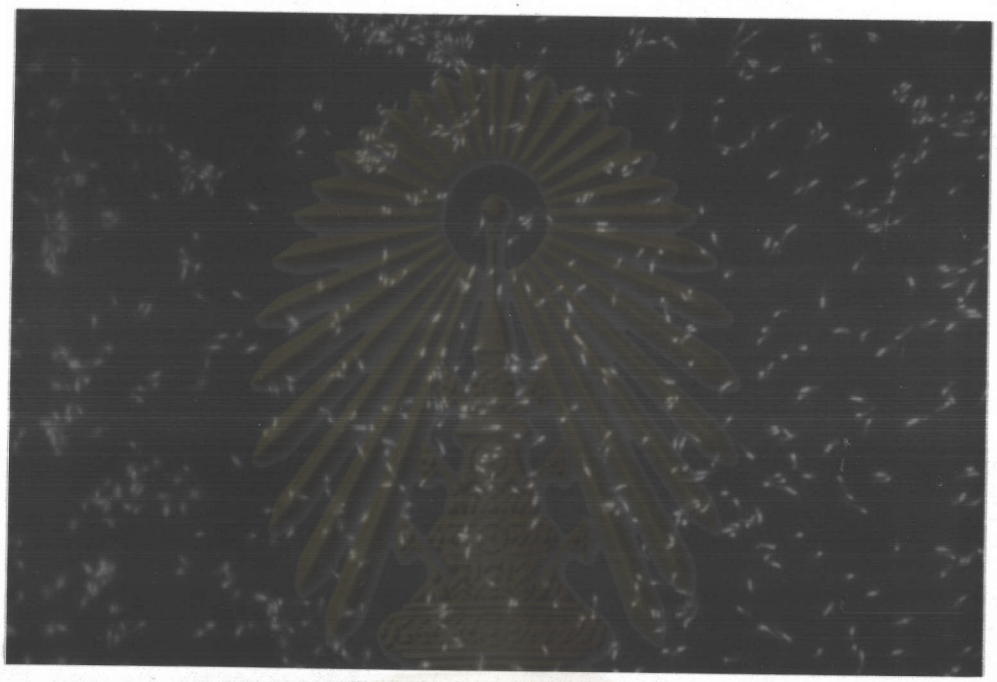


Figure 14. Fluorescent micrograph of R. japonicum.

Pure culture of R. japonicum, the slow growing rhizobium, was used as control for checking the cross reaction of the fluorescent antibody. The sample was primary stained with the fluorescent antibody and double stained with ethidium bromide.

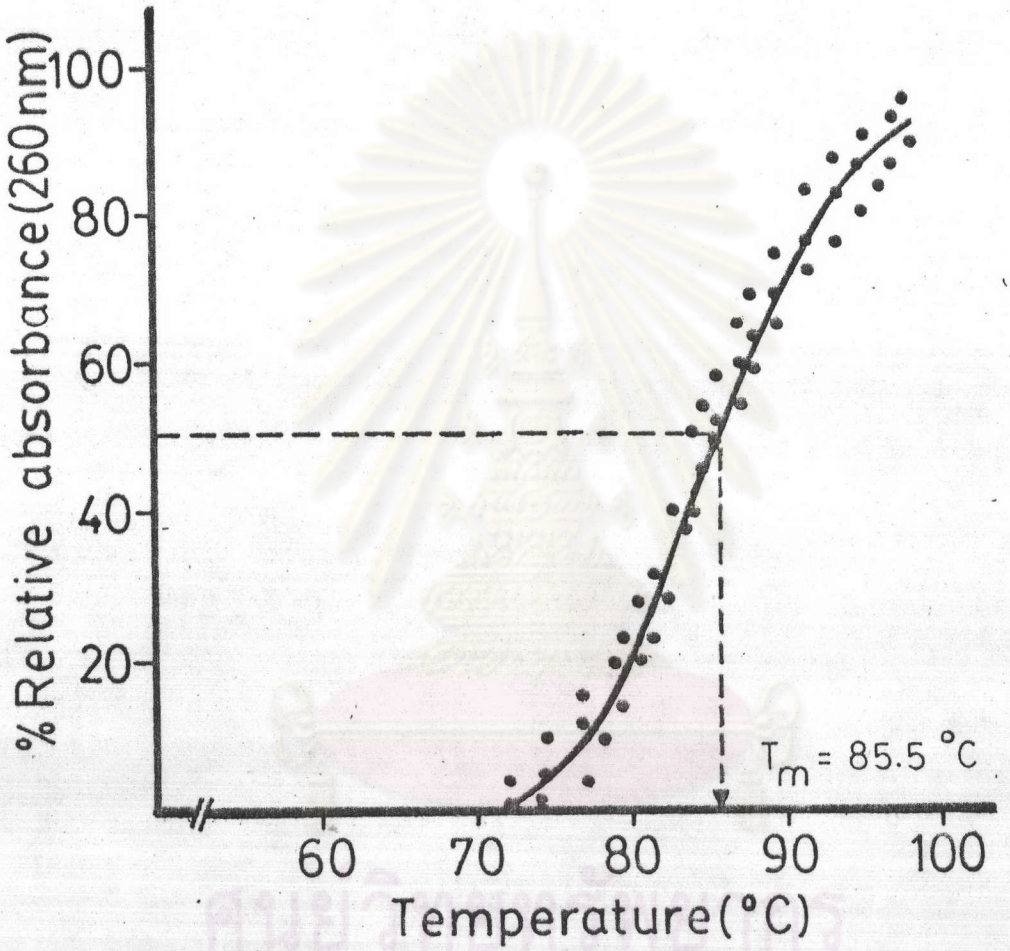
7. Measurement of the GC content.

To be assured that, strains being isolated were the genuine derivatives of WT, we should therefore, determined a % GC content between them. P_{19} was used as the representative. Its chromosomal DNA was isolated and purified in parallel to that of the WT. The procedures including the calculation of the % GC were as described in Methods section 7. Calf thymus DNA (Fig. 15) was employed as the standard DNA for a priliminary checking of the %GC and R. japonicum chromosomal DNA, isolated and purified under the same procedures, was used as the positive control. Data were presented in 3 different figures, Fig 16, 17 and 18. Based on our procedures, we found an identical value of % GC between WT and P_{19} chromosomal DNA (61.2%) which was significantly different from R. japonicum chromosomal DNA (64.9%).

8. Bioenergetic properties of WT and its mutants.

Generally, if two strains of bacterium provided a difference in properties such as growth yield and generation time, although they were grown in the same condition, then the point to be asked whether there was a defection occurred in the pathway of energy yielding. Hence, rate of O_2 consumption and level of ATPase activity in whole cell which being cultivated in the presence and the absence of salt were the next target to be explored. Summation of the result was shown was in Table 5 and the performance of the statistical analysis of these results were illustrated in Table 6 and 7. Details of the statistical analysis were described in Appendix 5,6. Based on this statistical treatment, it was ensured that, in the presence of salt, rate of O_2 consumption in the whole cells of each mutant was increased to 2-folds whereas the level of ATPase activity in all strains tested were remained at the same value.

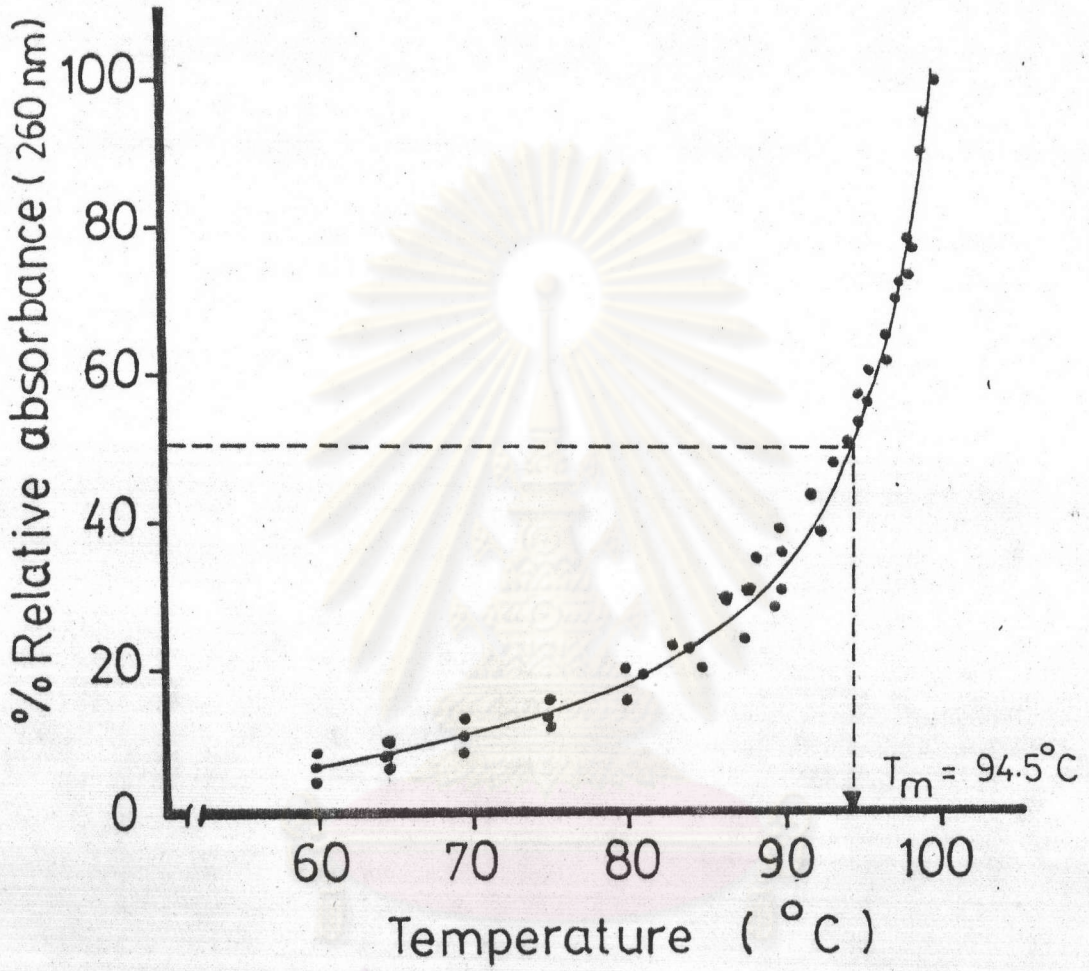
Figure15. The DNA melting curve of calf thymus



The DNA melting curve of calf thymus was performed as described in Methods Section 7. The Tm for calf thymus DNA was deduced, as 85,5°C from the melting curve. Therefore, % G + C of calf thymus DNA = (85.5 - 65.4) x 2.44 = 39.3%.

Three different melting curves represented triplicated experiments.

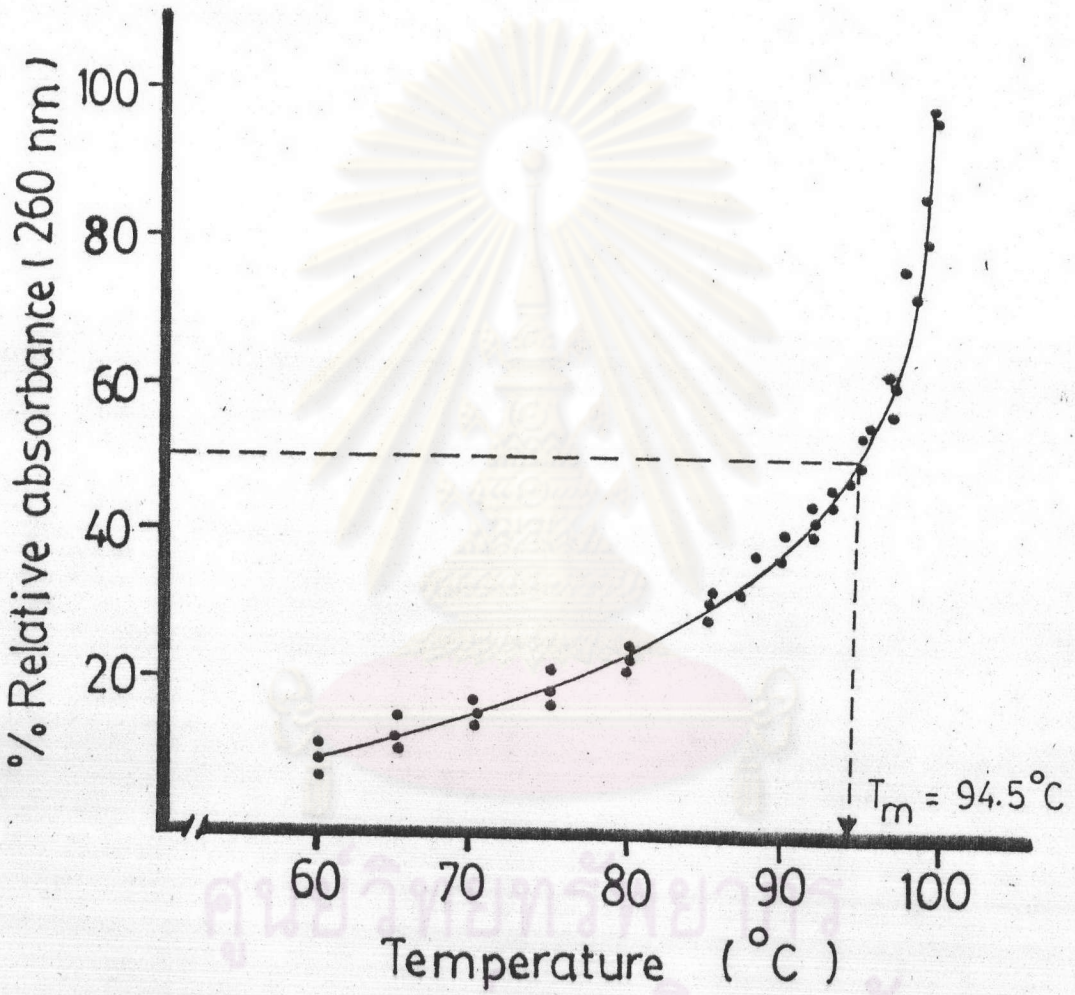
Figure16. The DNA melting curve of TAL 113



The hyperchromicity of TAL 113 DNA was registered at OD_{260} nm as% relative to that at 25°C with the function of temperature. The inflection point (Tm) for TAL 113 DNA was deduced, as 94.5°C from the melting curve. Therefore, % G + C of TAL 113 DNA = (94.5-69.4) x 2.44 = 61.2%.

Three different melting curves represented triplicated experiments.

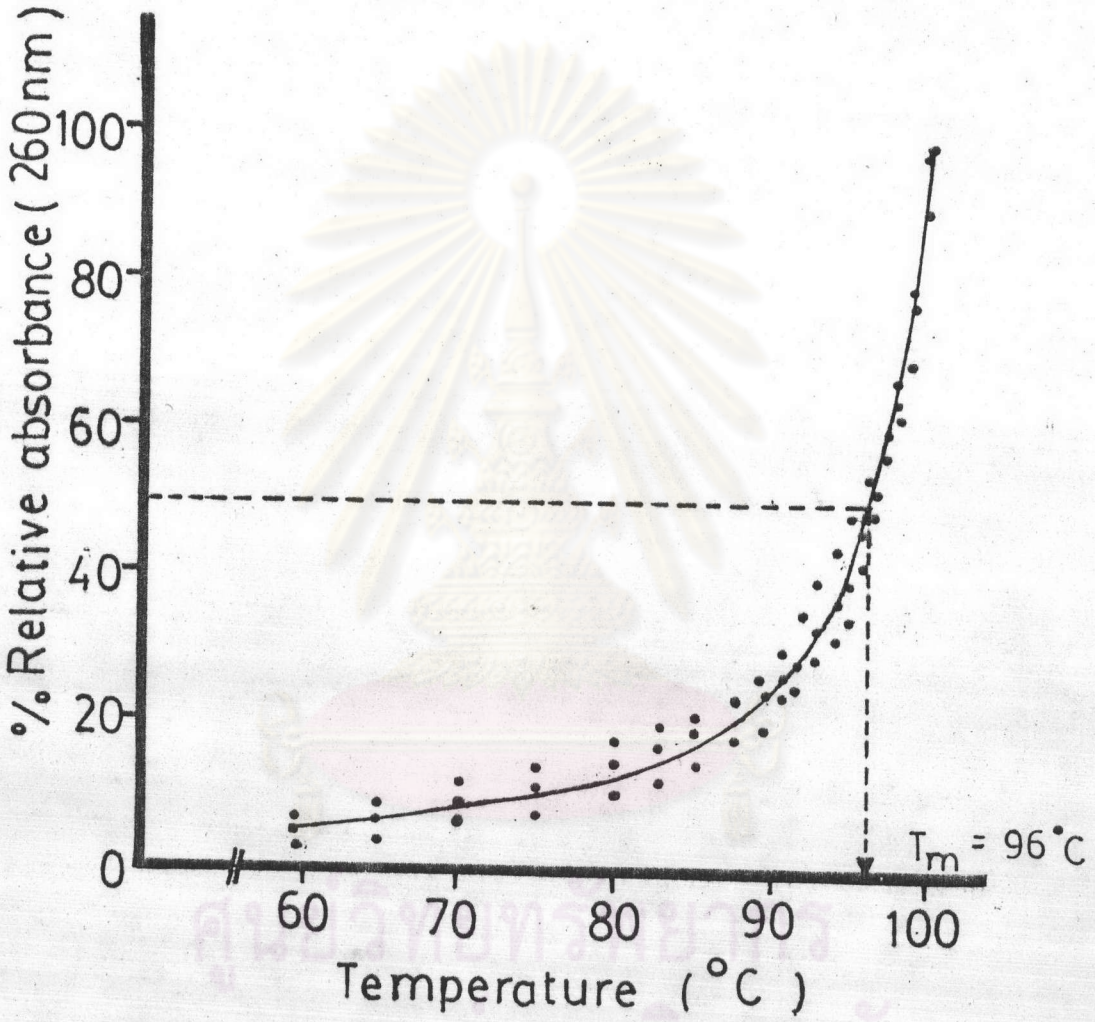
Figure 17. The DNA melting curve of mutant P_{19}



The DNA melting curve of Mutant P_{19} was performed as described under Figure 16. The Tm for Mutant P_{19} DNA was deduced as 94.5°C from the melting curve. Therefore, % G + C of Mutant P_{19} DNA = (94.5 - 69.4) x 2.44 = 61.2%.

Three different melting curves represented triplicated experiments.

Figure18. The DNA melting curve of R. japonicum



The DNA melting curve of R. japonicum was performed as described under Figure 16. The Tm for R. japonicum DNA was deduced as 96.0°C from the melting curve. Therefore, % G + C of R. japonicum DNA = (96.0 - 69.4) x 2.44 = 64.9%.

Three different melting curves represented triplicated experiments.

Table 4. Effect of high concentration of sodium ion on O_2 consumption and ATPase activity of WT TAL 113 and its mutants.

Strain no.	Cell grown in YM.		Cell grown in YM + 0.3 M NaCl	
	O_2 Consumption[1] (μmoles/min/mg protein)	ATPase Activity[1] (μmoles/min/mg protein)	O_2 Consumption[1] (μmoles/min/mg protein)	ATPase Activity[1] (μmoles/min/mg protein)
TAL 113	6.4 ± 0.9	2.2 ± 0.1	(-)	(-)
P_1	6.2 ± 0.5	2.7 ± 0.7	13.3 ± 2.4	3.4 ± 0.3
P_5	4.8 ± 0.6	2.7 ± 0.4	11.5 ± 2.1	3.5 ± 0.5
P_{19}	4.8 ± 0.4	2.7 ± 0.5	10.0 ± 0.6	2.7 ± 0.5
P_{21}	4.1 ± 0.3	2.6 ± 0.5	13.4 ± 0.1	2.3 ± 0.1

Cells at exponentially phase, grown either in YM medium or YM + 0.3 M NaCl, were used and the O_2 consumption and ATPase activity were carried out. Expression of the O_2 consumption and of activity of the ATPase were as described in the Methods section.

1 Each value was an average of 4 replicates.

(-) WT TAL 113 could not grow in YM + 0.3 M NaCl.

Table 5. Statistical analysis of salinity effect of sodium ion on O_2 consumption of WT TAL 113 and its mutants.

Strain no.	O_2 Consumption (μmoles/min/mg protein)		Statistical Decision among strains[1]
	Cells grown in YM	Cells grown in YM + 0.3 M NaCl	
TAL 113	6.4 ± 0.9	NG	
P_1	6.2 ± 0.5	13.3 ± 2.4	SD-
P_5	4.8 ± 0.6	11.5 ± 2.1	SD-
P_{19}	4.8 ± 0.4	10.0 ± 0.6	SD-
P_{21}	4.1 ± 0.3	13.4 ± 0.1	SD-
Statistical Decision between treatment	SD+	SD+	

NG = No growth

1 Based on the statistical analysis of F test (Fisher's test) with randomized complete block design, no significant difference among strains at the 0.05 level of significance was found.

2 Based on the statistical analysis of F test with randomized complete block design, there was significant differece between treatment with and without salt at the 0.05 level of significance.

The method of calculation was described in the Appendix 5.

Table 6. Statistical analysis of salinity effect of sodium ion on ATPase Activity of WT TAL 113 and its mutants.

Strain no.	ATPase Activity (μmoles/min/mg protein)		Statistical Decision among strains[1]
	Cells grown in YM	Cells grown in YM + 0.3 M NaCl	
TAL 113	2.2 ± 0.1	NG	
P_1	2.7 ± 0.7	3.4 ± 0.3	SD-
P_5	2.7 ± 0.4	3.5 ± 0.5	SD-
P_{15}	2.7 ± 0.5	2.7 ± 0.5	SD-
P_{21}	2.6 ± 0.5	2.3 ± 0.1	SD-
Statistical Decision[2] between treatment	SD-	SD-	

NG = No growth, SD- = No significant difference

1,2 The analysis of F test, taken either among strains or between treatment, showed no significant difference at 0.05 level of significance.

The method of calculation was described in the Appendix. 6.

9. Comparison of the rate of sodium efflux

9.1 Efffect of starvation and reconcilation on O_2 consumption.

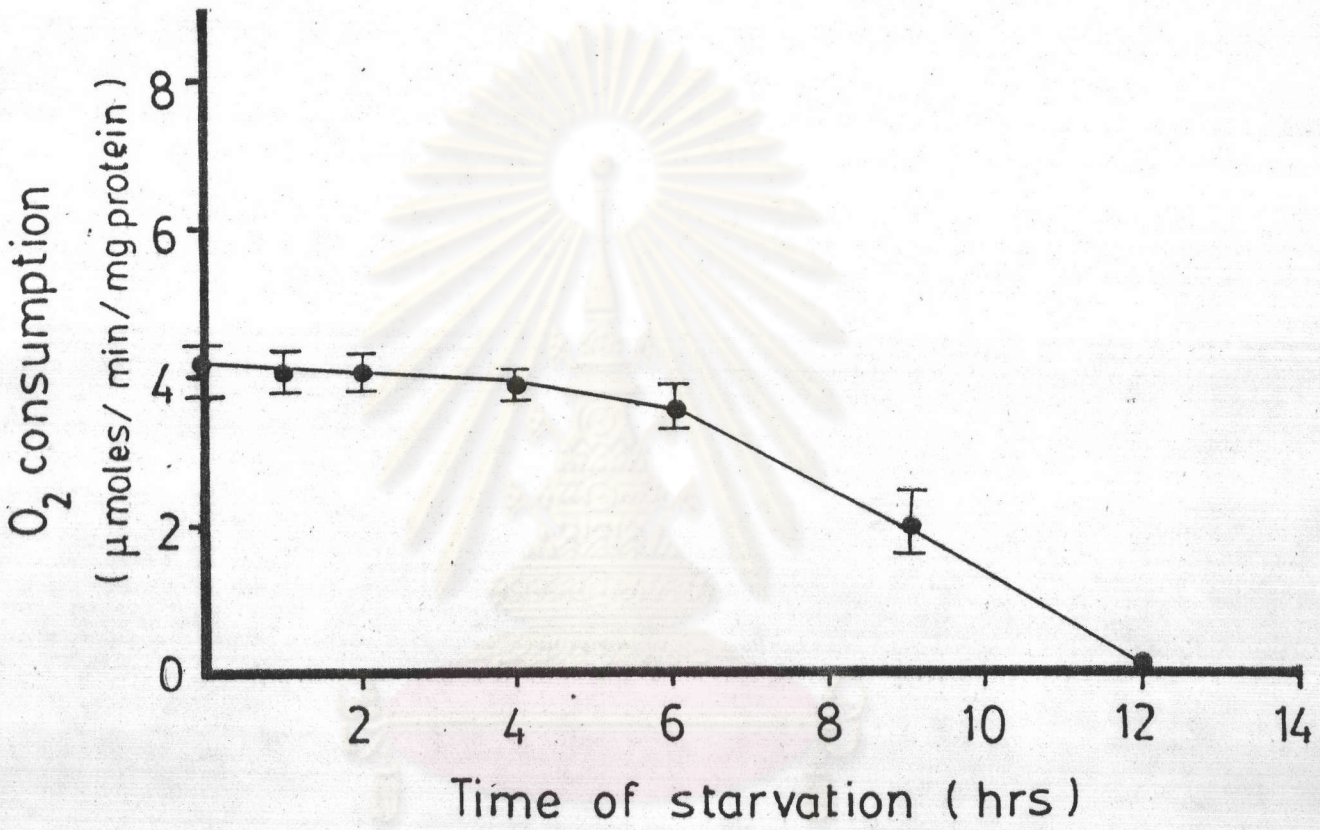
The hypothesis was proposed that an increment in rate of respiration was related to the drive of Na^+ out from the cells. To test the hypothesis, endogeneous storage of energy should be depleted. Thus a starvation experiment was performed. Fig. 19 showed a starvation profile of WT cell whereas Fig. 20 revealed those of mutant P_{19} in the presence and the absence of salt. It was found that, 12 hrs was the key of starving hour which cause the declination of O_2 consumption down to zero. After succinate was added, the O_2 consumption was immediately increased. The rate of O_2 consumption detected in the starved cells of mutant P_{19} was jumped to 2 folds when cells were cultivated in the presence of salt.

9.2 Measurement of sodium efflux.

Assay of sodium efflux was a rate measurement of a Na^+ excretion from intracellular to extracellular environment. Procedure used was as described in Methods section 10. Kinetic of sodium efflux of each strain was performed with an extreme precaution. Exact pattern of the experimental peformance was essentially important. In this experiment, starved cells were passively loaded with $^{22}Na^+$ until an equilibrium was reached and succinate was promptly added to drive the efflux of Na^+. Kinetics of this Na^+ efflux was followed for 10 min as shown in Fig. 22, 23. An initial rate of Na^+ efflux was calculated from the slope of the efflux profiles and summarized the obtained results in Table 7. FCCP and DCCD were the two uncoupling inhibitors being used to verify the test. Efflux of Na^+ in starved cells without

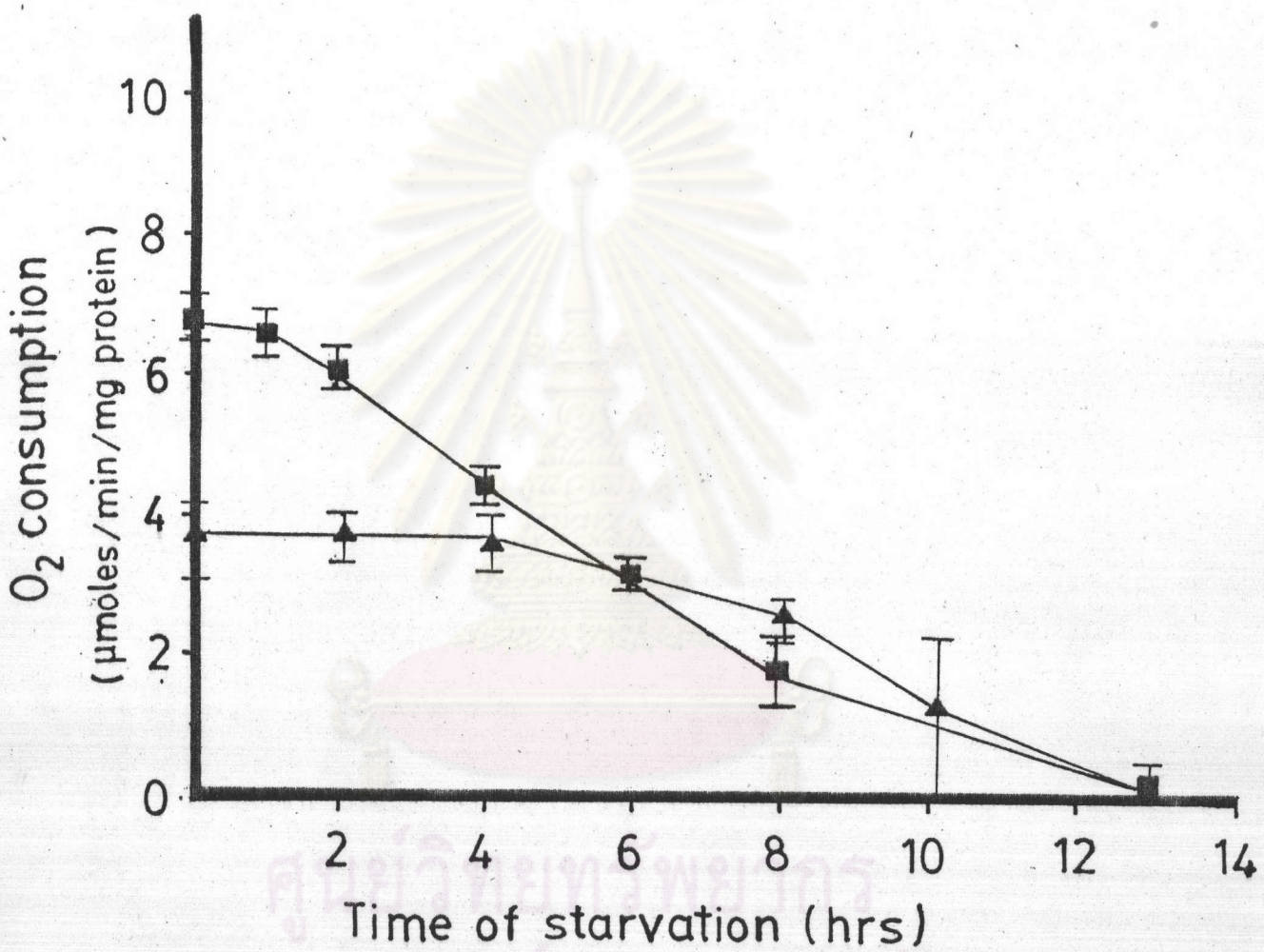
any addition of succinate was assumed as an endogeneous control and used to substract from those obtained with the addition of succinate. According to our treatment, a minus effect was found in the initial rate of all cases when FCCP was added in the assay. However, the initial rate of Na^+ efflux found in P_{19} were significantly higher than those obtained from the wild type regardless of growth conditions. Hence, cells cultivated in the salted condition caused nearly a duplication in the efflux of Na^+ as judged from and increase in values from 1.0 to 1.7 η mole per min per mg protein. An addition of DCCD caused no change in this trend.

Figure19. Starvation effect on the O_2 consumption of TAL 113



YM grown cells were used for starvation experiment. Cell peltet was resuspended in 0.1 M phosphate buffer pH 7.2 containing 0.01% $MgSO_4$. Starvation was generated by shaking the culture at 30°C. At different time intervals, a portion of the culture was taken as sample for O_2 consumption measurment.

Figure 20. Starvation effect on the O_2 consumption of mutant P_{19}

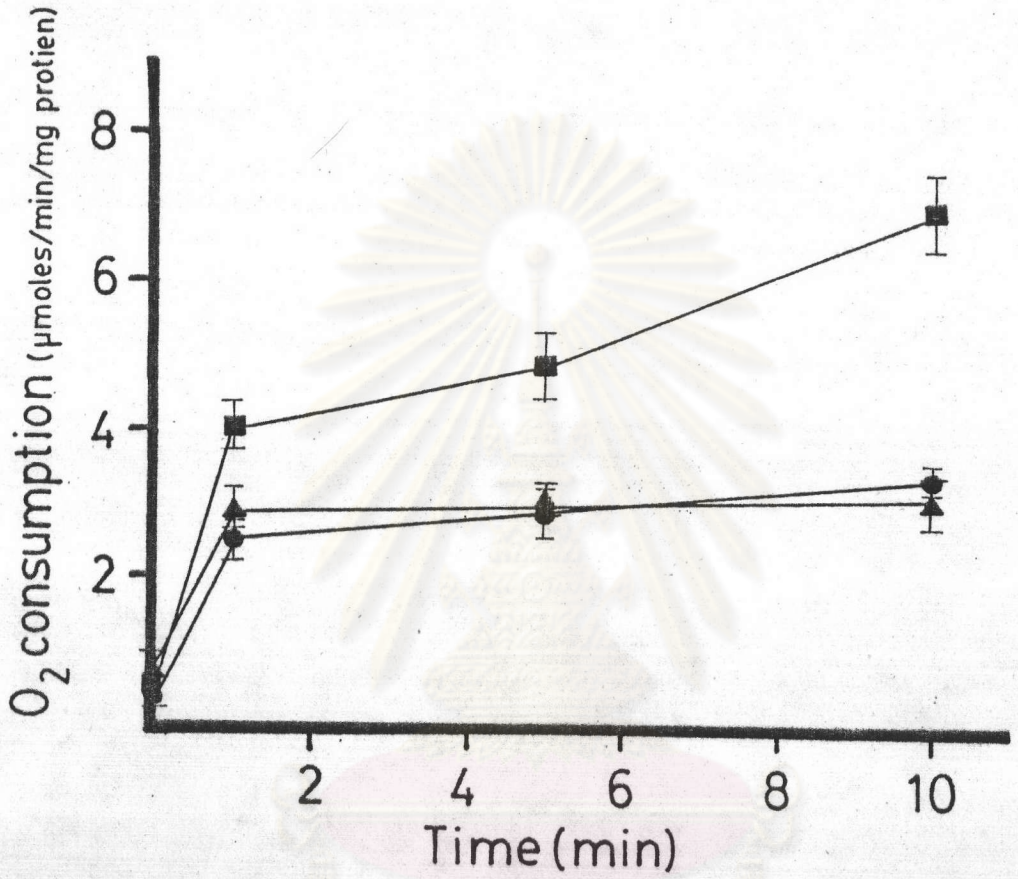


Growth of cultures used and method of cell starvation were performed as previously described under Figure 19.

▲——▲ Mutant P_{19} grown in YM before starving

■——■ Mutant P_{19} grown in YM + 0.3 M NaCl before starving.

Figure 21. The reconcilation of O_2 consump--tion



Wild type and mutant P_{19} cells, grown in YM with and without salt, were starved as previously described. After 12 hrs of starvation, 10 mM sodium succinate was added, and the O_2 consumption was measured.

●——● WT grown in YM before starving

▲——▲ P_{19} grown in YM before starving

■——■ P_{19} grown in YM + 0.3 M NaCl before starving

Figure 22. Measurement of sodium efflux in WT and mutant P_{19}

The starved cell was passively loaded with $^{22}NaCl$ and assayed for ^{22}Na content as described in the Methods.

●——● WT, 10 mM sodium succinate was added at zero time.

○——○ WT, no addition of sodium succinate

▲——▲ mutant P_{19} grown in YM before starvation, 10 mM sodium succinate was added at zero time.

△——△ mutant P_{19} grown in YM before starvation, no addition of sodium succinate

■——■ mutant P_{19} grown in YM + 0.3 M NaCl before starvation, 10 mM sodium succinate was added at zero time.

□——□ mutant P_{19} grown in YM + 0.3 M NaCl before starvation, no addition of sodium succinate.

Figure22. Measurement of sodium efflux in WT and mutant P_{19}

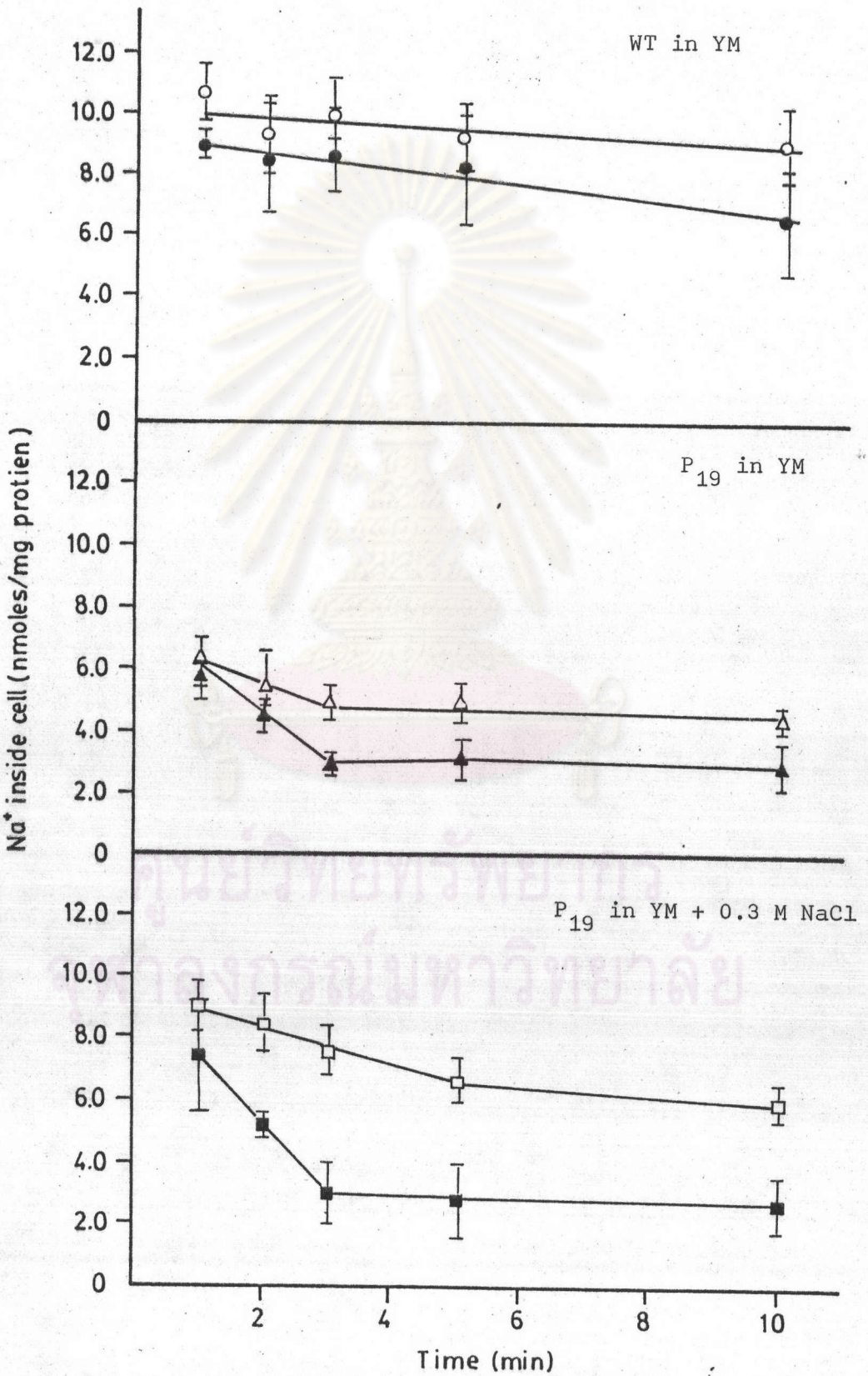


Figure 23. Effect of an addition of FCCP and DCCD on sodium efflux of WT and mutant P_{19}

The starved cell was passively loaded with $^{22}NaCl$ as described in Methods. 10 μM of FCCP or 40 μM of DCCD was added 3 min before starting of an assay.

o-----o WT with an addition of FCCP (10 μM)

●-..-● WT with an addition of DCCD (40 μM)

o——o WT as control

Δ----Δ P_{19} grown in YM before starvation with an addition of FCCP (10 μM)

▲-..-▲ P_{19} grown in YM before starvation with an addition of DCCD (40 μM)

Δ——Δ P_{19} grown in YM before starvation as control

□---□ P_{19} grown in YM + 0.3 M NaCl before starvation with an addition of FCCP (10 μM)

■-..-■ P_{19} grown in YM + 0.3 M NaCl before starvation with an addition of DCCD (40 μM)

□——□ P_{19} grown in YM + 0.3 M NaCl before starvation as control.

Figure 23. Effect of an addition of FCCP and DCCD on the sodium efflux of WT and mutant P_{19}

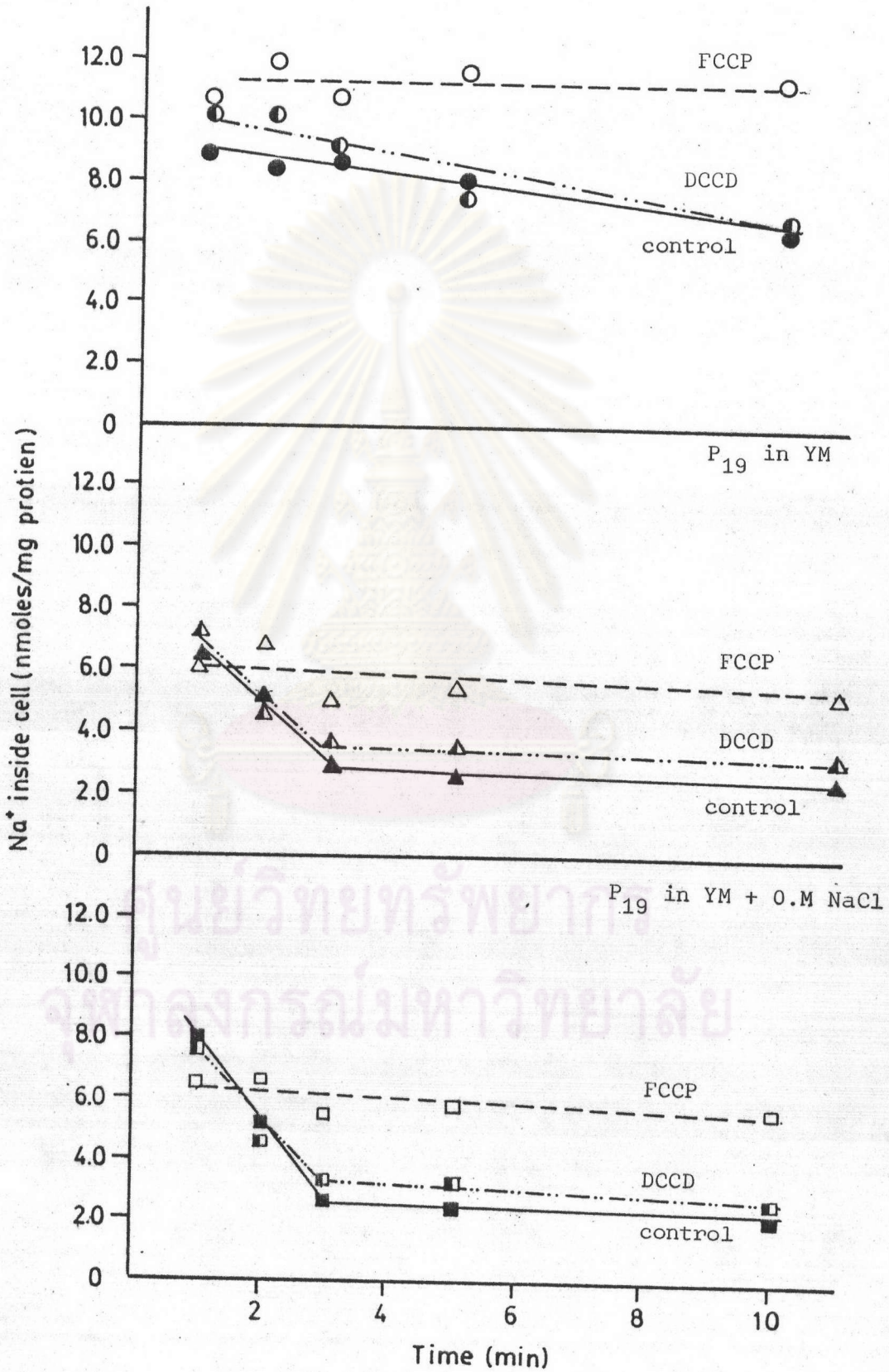


Table 7. Effect of an addition of FCCP and DCCD on the Na^+ efflux in WT and mutant P_{19}.

Strain	Rate of Na^+ efflux[1] (ηmole/min/mg protein)			
	- Succinate[2]	+ Succinate[3]		
		Control[4]	FCCP[5]	DCCD[6]
WT.	0.1	0.2	-0.1	0.3
P_{19} in YM.	0.6	1.0	-0.5	1,2
P_{19} in YM + 0.3 M. NaCl	0.5	1.7	-0.4	1.7

1. The initial rate of Na^+ efflux was calculated from Fig. 15 and 20.
2. Values of Na^+ efflux, assayed under no addition of succinate, were served as the endogeneous control.
3. Values shown were the difference of the Na^+ efflux assayed under addition of 10 mM succinate substracted from that of the respective endogenous control.
4. Values shown were the Na^+ efflux obtained in normal condition.
5. Values of this column were the Na^+ efflux assayed under an addition of FCCP.
6. Values of this column were the Na^+ efflux assayed under an addition of DCCD.