BIOCHEMICALLY ACTIVE SUBSTANCES FROM ACTINOMYCETES AND OTHER ORGANISMS. II

EFFECTS OF VARIOUS COMPOUNDS ON MOTILOMETRY

HARUYOSHI AZUMA and HIROMI B. MARUYAMA

Department of Microbiology and Chemotherapy
Nippon Roche Research Center
200 Kajiwara, Kamakura-247, Japan

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Effects of various compounds known to affect the bacterial or protozoan motility on the motility of *Escherichia coli* S-26 were examined by a newly-devised simple method, motilometry. Under catabolite repression, adenosine 3′, 5′-cyclic phosphate as well as theophylline was found to stimulate the motility in this system. On the other hand, some alkaloids like eseline, strychnine and atropine, membrane-active compounds such as phenethyl alcohol and levallorphane, respiration inhibitors or several metals showed a strong inhibition at a concentration causing no or little growth inhibition.

In the previous paper[1], we established a simple and quantitative assay method for the motility of *Escherichia coli* in a thin glass tube in an attempt to screen for motility inhibitors or stimulators from cultured broth of actinomycetes and other microorganisms. In this method, *E. coli* Strain S-26 inoculated at the top of a tube filled with motility medium were allowed to migrate towards the tip and the motility was quantified by the distance from the inoculated position to the spearhead of migration.

The present paper reports the effects of various compounds known to affect the motility of various organisms and adenosine-3′, 5′-cyclic phosphate metabolism on the motility of *E. coli* S-26. The new assay system, motilometry, was examined in order to obtain a perspective on possible usability of this method for screening.

Material and Methods

Bacterial Strain and Cultural Conditions

Escherichia coli strain S-26[1] was used throughout the experiment. Cultural conditions used for preculture and the migration culture in the motility medium (MM) were the same as described in the previous paper[1]. Repression medium (GMM) contained 0.04 M glucose.

Motility Measurement

Test compounds were mixed with hot MM and packed in a sterilized glass tube as described previously[1]. Incubation was done under standard conditions in a vertical position[1]. The distance of migration in mm from the inoculated position to the two bands observed in some cases (Bands I, II) or the spearhead point of turbid area was determined. The percent inhibition or stimulation was calculated from the distances of migration with test compounds and the control.

Chemicals

Adenosine-3′, 5′-cyclic monophosphate (cAMP) was purchased from Daiichi Chem., Tokyo. Penicillin G and chloramphenicol were generous gifts from Dr. T. TAKEUCHI, Institute of Microbial Chemistry, and from Sankyo Co., respectively. Levallorphane (1, 3-hydroxy-N-allyl-

morphinane) tartarate was a product of F. Hoffmann-La Roche AG. Basle. Other chemicals used were purchased mostly from Wako Chem. Co., Ltd., Tokyo.

Results and Discussion

1. Repression of Motility by the Addition of Various Sugars

The presence of 0.01~0.1 M glucose inhibited the motility of strain S-26 depending upon the concentration. Glucose-containing media caused the formation of characteristic bands (I and II) around the spearhead of the migration[1]. Table 1 shows the effect of other sugars or related compounds on the motility by the present method. As is clearly seen, sugars such as galactose, maltose, lactose and deoxyribose or sodium lactate and pyruvate caused a similar inhibition of the motility, while sucrose, ribose and glycerol showed no effect. It was noted that, in most cases, the reduction in motility corresponded to the increase in growth rate, suggesting a close relation of motility to the utilization of sugars and hence the motility inhibition of the catabolite repression type. This fact is generally consistent with the finding of BUETTNER *et al.*[2] on the relationship between catabolite repression and cAMP where carbon sources that rather poorly supported growth gave higher intracellular concentration of cAMP than did sugars that supported rapid growth.

Table 1. Effect of sugar on motility and growth of *E. coli* S-26

Sugar	Conc. (M)	Relative motility (%)		Growth doubling time* (min.)
		Band II	Band I	
Non sugar-MM (control)		—**	100.0	61
Glucose	0.05	56.2**	80.0***	43
Galactose	0.05	(59)+	78.9	44
Fructose	0.05	64.5	94.6	52
Deoxyribose	0.05	—	41.7	60
Ribose	0.05	—	104.3	54
Glycerol	0.05	—	101.8	49
Na-Pyruvate	0.05	66.2	92.1	49
Na-Lactate	0.05	+	92.5	50
Sucrose	0.05	—	95.3	64
Maltose	0.05	+	82.8	47
Lactose	0.05	(70)+	95.0	48

* Doubling time was obtained from a shaking culture in MM minus agar by the use of a MONOZ type tube, followed by turbidity.

** Band II was not formed.

*** Relative motility: percent of the migrated distance to that in MM (Band I of control=100 %).

\+ The formation of Band II was not quite reproducible.

Also noticeable is that Band II was not formed in the presence of sucrose, maltose, ribose, deoxyribose, glycerol and lactate. The reason for this phenomenon remain to be clarified as well as the inhibition by various sugars.

2. Effect of Vitamins and Amino Acids on the Motility

The effects of vitamins and amino acids on the migration of Bands I and II in GMM are summarized in Table 2. It is shown that none of those listed gave any significant inhibition or stimulation on motility in this method, when added to GMM at a concentration of 1 mM. It is known that some amino acids like serine and asparagine are potent chemotactic attractant of *E. coli*[3] in a synthetic semi-solid medium. However, in the present system, since the motility was examined in a rich medium such as GMM containing various amino acids originated

from casein and yeast extract, it is quite reasonable that an extra-supplmentation of amino acid causes no positive effect on the motility. This fact in turn provides an advantage in that any motility stimulator selected by this method would not be active by virtue of chemotactic events mediated by chemotactic receptor sites for amino acids[4].

Table 2. Effect of vitamins and amino acids (L-) on relative motility

Compound (1 mM)	% Migration		Compound (1 mM)	% Migration	
	Band I	Band II		Band I	Band II
Control	100	100	Cysteine	98.3	96.7
Glycine	97.0	95.2	Methionine	95.8	92.4
Alanine	96.2	94.3	Phenylalanine	97.9	95.2
Valine	98.3	97.6	Tyrosine	94.9	92.9
Leucine	94.1	91.9	Tryptophane	91.5	93.9
Isoleucine	99.2	97.6	Thiamine-HCl	99.6	100.0
Serine	84.7	91.0	Roboflavin	105.9	105.0
Threonine	81.7	89.2	Pyridoxine-HCl	98.1	98.3
Aspartic acid	93.6	93.3	Ascorbic acid	99.6	99.5
Glutamic acid	96.2	95.2	Nicotinic acid	100.0	102.1
Proline	102.5	100.5	Biotin	101.5	101.3
Arginine	97.0	97.1	Calcium-D-pantothenate	98.1	99.2
Histidine	91.5	90.5			
Lysine	98.7	93.8			

3. Effect of Various Compounds Known to Affect the Motility in Other Systems on the Motility Observed in the Present System

Table 3 demonstrates the effect of various compounds on the motility in this system. It was found that such alkaloids as atropine and strychnine showed a strong inhibition (40~50 %) at 10^{-3} M. The inhibitory effect of colchicine known to depolymerize the microtubule structure[5] was not marked in comparison with the others mentioned above. It should be noted that the inhibition percent on Bands I and II was significantly different from each other in some cases, and that, in cases where a srong inhibition was found on Band I, no formation of Band II was in general observed. Table 3-a shows that membrane-affecting compounds such as levallorphane and phenethyl alcohol were found to strongly inhibit the motility. The primary point of action of these compounds is known to be damage of the plasma membrane which causes permeability changes, instability of the surface and changes in phospholipid composition that, all together, eventually result in inhibition of nucleic acid synthesis, breakdown of ATP and so forth[6,7]. Since the minimal inhibition concentration (MIC) of levallorphane on growth of S-26 was found to be 2 mg/ml by the broth dilution method, the 50 % inhibition concentration of motility (110 μg/ml) was approximately one twentieth of the MIC and roughly equivalent to the concentration causing spheroplast lysis of *E. coli* strain K12 as reported by FROMAGEOT *et al*[8]. Similarly, phenethyl alcohol caused motility inhibition at a concentration far lower than the lethal dose. As can be seen, some detergents and antibiotics attacking the cell surface also inhibited motility in this system (Table 3, a and c). However, even such antibiotics like chloramphenicol and tetracycline that are known to inhibit protein synthesis also showed a strong motility inhibition at a concentration far less than their MIC values (Table 3-c). This fact may suggest the involvement of some specific protein synthesis in cell movement as assayed by the present system. It also indicates the possible use of the motilometry as a sensitive assay method

for certain types of antibiotics.

That all respiration inhibitors tested uninformly showed a strong inhibition at a concentration causing little growth inhibition (Table 3-b) indicated a close relationship between the energy generating system and motility. Similar effects of these compounds were observed with *Pseudomonas fluorescens* by FAUST and DOETSCH[9]. Since no ATPase activity was found in bacterial flagellar filaments[10], the energy for flagellar movement may be directly or indirectly mediated by the membrane where the respiratory chain and oxidative phosphorylation systems are thought to be arranged. Regarding the mechanism of the movement, there are few reports relating to the concrete events. According to a speculation of VAIRUZIS and DOETSCH, membraneous locomotion was considered to give the force for the movement[11]. From the present

Table 3. Effect of various compounds on the relative motility and growth

(a)

Compound added	Conc. (M)	% Migration		Growth-doubling time (min.)
		Band II	Band I	
Control (GMM only)		100	100	48
Acetylcholine	10^{-3}	69.6	80.3	48
Sodium barbital	10^{-3}	69.6	63.7	48
Atropine sulfate	10^{-3}	60.1	54.0	48
Eseline sulfate	10^{-3}	60.1	53.3	48
Strychnine sulfate	10^{-3}	—	47.8	48
Colchicine	10^{-3}	95.5	85.3	48
β-Phenethyl alcohol	10^{-3}	36.5	40.0	48
Levallorphane	10^{-3}	—	20.0	ND*
Triton X-100	1 mg/ml	76.3	74.1	48
Sodium dodecylsulfate	5×10^{-4}	—	12.5	48
Cacodylic acid	10^{-3}	96.6	73.7	48
Sodium fluoride	10^{-3}	97.8	97.0	48
EDTA	5×10^{-4}	—	4.3	ND*
Lysozyme	20 μg/ml	99.5	99.3	ND

(b)

Compound added	Motility inhibition		M.I.C.***	$\frac{ID_{50}}{M.I.C.}\times100$ (%)	$\frac{ID_{90}}{M.I.C.}\times100$ (%)
	ID_{50}**	ID_{90}**			
Potassium cyanide	0.20 mM	1.00 mM	8.0 mM	2.5 %	12.5 %
Dinitrophenol	0.05	0.10	2.0	2.5	5.0
Sodium azide	0.015	0.40	3.0	5.0	13.3

(c)

Chloramphenicol	0.70 μg/ml	1.0 μg/ml	5.0 μg/ml	14.0 %	20.0 %
Tetracycline	0.22	0.40	5.0	4.4	8.8
Penicillin G	7.0	12.0	50.0	14.0	24.0
Novobiocin	20.0	40.0	200	10.0	20.0
Polymyxin B	0.22	0.80	2.0	11.0	40.0

* ND: not determined.
** ID_{50} and ID_{90}; the concentration exhibiting 50 % and 90 % inhibition of motility.
*** MIC, the minimal inhibition concentration of growth determined by agar-dilution method.

results, specific inhibition of energy generation may be speculated to bring about a strong inhibition of motility.

Effects of the addition of metals on the motility are shown in Table 4. A strong inhibition was found in cases with 10^{-3} M concentration of Cu^{2+}, Hg^{2+}, Co^{2+}, and Ag^{+}.

Table 4. Effect of metal on bacterial motility

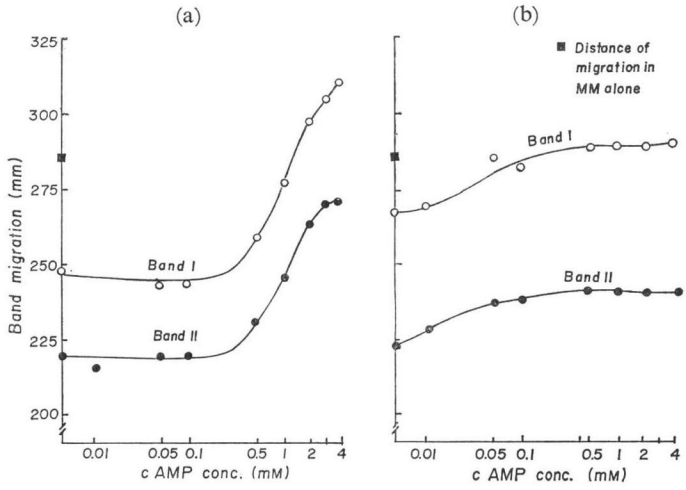
Compounds	Conc. (M)	% Migration		Growth doubling time (min.)
		Band II	Band I	
Control		100	100	40.5
$CuCl_2$	10^{-4}	—*	7.5	41
$AgNO_3$	10^{-4}	—	0.4	No growth
	2×10^{-5}	57.9	63.3	41
$HgCl_2$	10^{-5}	—	4.2	No growth
	5×10^{-6}	—	12.4	41
$CoCl_2$	5×10^{-4}	43.8	57.4	115.5
$KCrO_4$	5×10^{-4}	—	50.7	42
$ZnSO_4$	10^{-3}	60.5	69.8	40
$PbCl_2$	10^{-3}	69.9	52.5	41.0
$BaCl_2$	10^{-3}	—	42.3	121.5
$CaCl_2$	10^{-3}	70.1	78.1	—**
$MgSO_4$	10^{-3}	80.2	89.4	—
LiCl	10^{-3}	94.6	93.6	—
$FeSO_4$	10^{-3}	95.8	97.0	—
$FeCl_3$	10^{-3}	97.0	94.7	—
$MnCl_2$	10^{-3}	112.6	102.3	—
$(NH_4)_6Mo_7O_{24}$	10^{-3}	92.8	94.7	—
NaCl	10^{-2}	98.4	96.4	—
NH_4Cl	10^{-2}	102.8	102.1	—

* Not observed. ** Not determined.

4. Reversal of the Motility Inhibition by cAMP

Cyclic AMP reversed the repression in motility caused by glucose or other sugars in this system, confirming the results reported previously by other methods[12,13]. Fig. 1-a demonstrates that a migration greater than in MM was observed in the GMM to which more than 1 mM cAMP was added. In the range from 0.1 to 3 mM, the migration distance of Band I increased as a function of cAMP concentration. On the other hand, the repression of motility by lactose was reversed only to the non-repression value (Fig. 1-b) although the reversal appeared at lower concentrations of cAMP (0.01~0.05 mM) than those in GMM. When the reversed distance (migration in MM minus that in sugar-containing MM) is double-reciprocally plotted as the function of cAMP, a linear relationship is seen as shown in Fig. 3. The apparent Km of cAMP was between $1.6\sim2.5\times10^{-3}$ M for glucose, deoxyribose and maltose but was smaller for galactose (4×10^{-4} M).

Fig. 1. Reversal of the repressed motility by cAMP. The migration distance of Bands I and II are plotted as the function of cAMP concentration integrated in MM containing 50 mM of glucose (a) or lactose (b).



The effects of theophylline and sodium fluoride were examined in this connection. Both

Fig. 2. Double reciprocal plot of the recovered distance (normalized) with respect to cAMP. Vo is the normalized distance in the presence of 50 mM sugar.

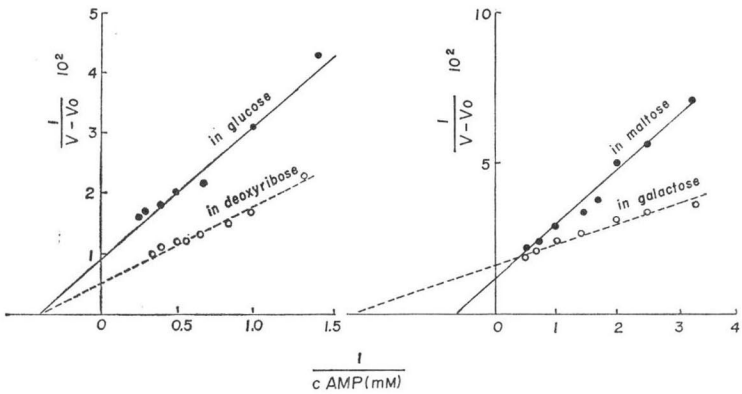
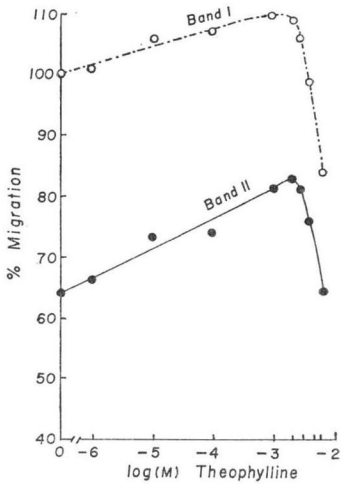


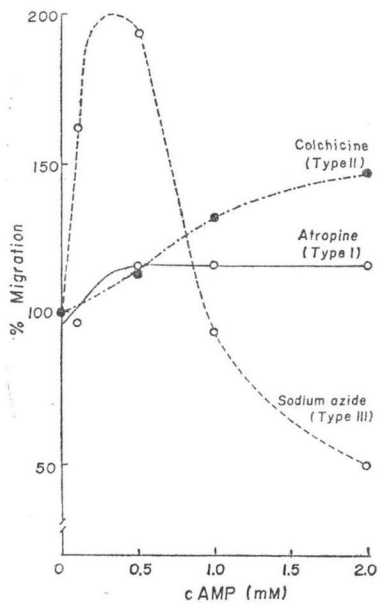
Fig. 3. Effect of theophylline on the motility. The migration percent of Bands I and II in GMM (no addition in GMM=100 %).



are known to elevate cAMP level in the animal system either by inhibition of cAMP phosphodiesterase[14] or by activation of adenyl cyclase[15]. Fig. 3 shows that theophylline has a marked reversing effect on repressed motility at concentrations less than 5 mM. However, a strong inhibition of motility is seen at higher concentrations. On the other hand, sodium fluoride gave no significant reversing effect (Table 3-a), which is consistent with the fact that it does not activate bacterial adenyl cyclase[16]. Although the precise mechanism of theophylline action remains to be clarified, it is conceivable that theophylline elevates the endogeneous cAMP level by inhibiting the degrading enzyme[15].

Fig. 4 shows the reversing effect of cAMP on the motility inhibition caused by various compounds. It is clear that there are three distinct types of reversal pattern. The reversal of atropine inhibition represents Type I in which the motility inhibited by atropine did not recover by increasing addition of cAMP. Strychnine, tetracycline and SDS were found to belong to this type. Type II is the case where the inhibition was completely reversed by the addition of a sufficient concentration of cAMP: colchicine, phenethyl alcohol, dinitrophenol and cyanide showed this type of reversal. Type III consists of the pattern in which the reversal was obtained at a certain concentration of cAMP but, at a higher concentration of cAMP, it was inhibitory. This type was seen with sodium azide and penicillin G. No explanation can be given at present for this phenomenon, although the difference in the type of pattern may be associ-

Fig. 4. Differential reversal by cAMP of the motility inhibition caused by various compounds. Percent migration at 0 concentration of cAMP is 30 %, 63 % and 30 % to the no-addition control for 5 mM atropine, 10 mM colchicine and 0.1 mM NaN_3, respectively.



ated with their different mechanism of inhibition.

5. Usability of the Motilometry for Screening

The effect of various compounds known to inhibit motility of various organisms by various methods were compared by this method. The results obtained showed that most of such compounds as neurotransmittance blockers, respiration inhibitors, microtubule depolymerizers and membrane-active substances and some metals exhibited inhibitory activity in the present system at a concentration causing no growth inhibition of *E. coli*. Chemotactic activity of certain amino acids was eliminated in this system by the presence of various amino acids present in casein and yeast extracts. The assay method, motilometry, is considered to be interesting as a simple screening system for compounds of the types discussed above, provided that the presence of heavy metals are taken into the consideration. Characterization of a unique substance obtained from the culture broth of a *Streptomyces* by this method will be reported in the subsequent paper[17].

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