Chemical Stimulation and Inhibition of Bacterial Motility Studied with a New Method.* (19148)

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In a recent electron microscope study of purified flagella from *Bacillus brevis* it was shown that they are composite protein structures with a trypsin-resistant axial filament and a trypsin-sensitive peripheral sheath (De Robertis and Franchi)(1,2). The axial filament has a highly complex helical ultrastructure that seems to be particularly well adapted to the mechanism underlying the motility of bacteria flagella. By studying the action of different chemical agents on this protein it was found that adenosintriphosphate (ATP) causes the contraction of the flagellar filaments while other organic and inorganic phosphate compounds have no visible influence. Furthermore, it was observed that the thiol inhibitors—p-chloromercuribenzoate and iodosobenzoate—interfere with the action of ATP(2).

These findings suggested the study of the action of these and other chemical agents on the motility of the living bacteria. The problems involved in bacterial locomotion have been recently discussed by Weibull(3). Fleming *et al.*(4.5) observed that in bacteria grown in sub-lethal concentration of penicillin motility is apparently influenced by radiant heat. However, the source of energy and the probable enzymatic mechanisms involved in bacteria locomotion were not investigated. In the present study several chemical agents known to act on specific groups present in proteins or on particular enzymatic processes were used. Special care was taken to follow the action of different drugs added to groups of bacteria previously observed in normal locomotion. For this purpose a special chamber and culture technic was developed. This method allows for prolonged and undisturbed observation of a group of bacteria while they are submitted to the action of different agents.

Material and methods. Proteus vulgaris, H × 19 was grown on semisolid agar to increase the motility and then transferred to plain agar the day before the test. The culture chamber was prepared as follows: Grooved slides were made by sticking down 2 cover-slip strips on a slide leaving between them a channel, 3-5 mm wide (Fig. 1). Cellophane strips (24 × 28 mm) were sterilized by boiling and afterwards kept in a Petri dish with sterile broth. To set a chamber, a thin film of a 24-hour culture of *P. vulgaris*

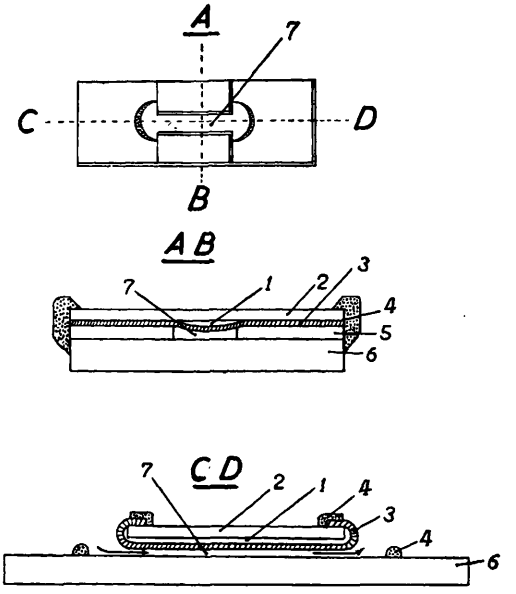

FIG. 1. Diagram of the culture chamber. *Upper,* view from above, *middle* and *lower* sections into lines A B and C D (see description in the text.) 1. culture, 2. cover-slide, 3. cellophane membrane, 4. wax, 5. cover-slide strip, 6. slide, 7. channel.

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TABLE I. Action of Different Inhibitors on the Motility of *Proteus Vulgaris* HX 19.

Inhibitor	Molar concentration	Degree inhibition	Time starting inhibition, sec	Time of max inhibition, sec
p. chloromercuribenzoate	.001	4+	30	180
Mercury dichloride	.01	4+	30	60
Etilmercurithiolsalicilate "merthiolate"	.025	3+	30	180
Iodosobenzoate	.002	3+	30	300
Iodoacetamide	.01	2+	30	90
Iodoacetate	.01	1+		180
Potassium cyanide	.001-.01			
Sodium azide	.01			
Malonate	.01			
Sodium fluoride	.01			
Chloroform	.08	3+	30	60
Chloral hydrate	.3	4+	20	60
" "	.14	3+	40	180
" "	.06	2+	40	180
Barbital	.33	3+	20	240
Phenobarbital	.1	3+	15	30
Metadon	.03	3+	30	120

1+, translation slowed down, vibration persists; 2+, a few bacteria with translation, vibration persists; 3+, translation stopped, some vibration *in situ;* 4+, total paralysis.

was spread over the middle zone of a sterile cover-slip and then kept in a Petri dish until it dried out. A cellophane strip was stretched cross-wise on top of a slide, and a loopful of melted plain agar was dropped on its center. Then the cover-slip, carrying the culture, was quickly inverted over it. After setting, the cellophane flaps were turned up on both ends and the chamber taken off from the supporting slide and laid, with the cellophane down, over the grooved slide. The chamber was pressed down with a piece of filter paper and, as soon as the sides were dry, was sealed with wax leaving both ends of the channel open. To prevent the spreading of the solution on the slide a narrow ring of wax was put around each open end of the channel. The inoculated chambers were kept moist in a Petri dish, over a layer of damp cotton. After 24 hours incubation at room temperature, the channel was filled with saline, and the chamber examined under a dry objective (N.A. 0.85) with the phase-contrast microscope. A place where the bacterial density was optimal and with no liquid currents was selected for the experiments. In some chambers it was possible to find small "swimming pools" with a few motile organisms that could be followed individually. The solution to be tested was dropped at one end of the channel and it ran easily by capillarity to the opposite end. The rate of flowing could be increased by absorbing the emerging liquid with a piece of filter paper. The drugs were generally dissolved in buffer phosphate of pH about 7 at the concentrations indicated in Tables I, II, and III. All together about 100 chambers were examined and submitted to the action of different drugs. In some cases only one substance was investigated in a single chamber but other times the action of two or more drugs were successively applied to the same group of bacteria.

Results. Action of different inhibiting drugs on the motility of Proteus vulgaris. Table I summarizes the main results obtained with different inhibitors on the motility of *Proteus vulgaris.* The degree of inhibition was estimated by the changes in motility until complete paralysis occurred. Although there were differences from one culture to another, probably related to variations in the chamber, age of the bacteria and so forth, in general the inhibition of motility followed quite a definite pattern. For example, with chloral hydrate (0.3 *M*) the first reaction was observed after 10 to 20 seconds when the movement of translation in the long forms of bacteria was diminished and finally stopped. A few seconds later translation of the medium size and short bacteria was also inhibited though they still showed vibration *in situ.* Finally a complete inhibition of motility was observed.

TABLE II. Reversal of Inhibition Produced by Thiol Inhibitors on the Motility of *Proteus Vulgaris* X 19.

Thiol inhibitor	Degree of inhibition	Thiol-reducing agents, M .005	Time starting to act, sec	Time max reversal	Degree of reversal
p. chloromercuribenzoate M .001	4+	Glutathione	20	60	Complete
		Cysteine	30	60	"
		Ascorbic acid			
Mercurichloride M .001	4+	Glutathione	30	180	Almost complete
Etilmercurithiosalicilate M .025	3+	"		90	Complete
Iodosobenzoate M .002	3+	Cysteine	60	150	"
Iodoacetamide M .01	2+	Glutathione			"
Iodoacetate M .01	1+	"		240	"

TABLE III. Action of ATP and Other Phosphate Compounds After the Inhibition of Motility with M .3 Chloral Hydrate for 60 Seconds.

Exp. No.	Treatment	Starting vibration, sec	Starting translation, sec	Starting vibration, sec	Starting translation, sec
11	Saline		300		150
29	Buffer pH 7	360		40	
30	Glucose 1-6 phosphate	240		120	
13	Glucose 1-6 phosphate	300		120	180
18	3 phosphoglyceric acid	120	180	80	150
18 bis	Sodium pyrophosphate	120	300	90	220
17	Buffer pH 7	120		40	
53	Na pyrophosphate	120	360		220
59	3 phosphoglyceric acid	90	540	120	300
55	Glucose 1-6 phosphate	150	270	120	210
83	Saline	210	360	40	90
92	"		600		300
97	"		510		360
Means		183	380	85	218

Action of thiol inhibitors. Table I shows that the strongest inhibiting action was observed with the mercaptide-forming agents: p-chloromercuribenzoate acting at a concentration 0.001 *M* and mercuric chloride at 0.01 *M*. Ethyl mercurithiosalicilate (Merthiolate), probably acting by a similar mechanism, produced also a considerable inhibition, although not complete, at a concentration of 0.025 *M*. The oxidizing agent iodosobenzoate had less inhibiting action, while the SH alkylating drugs, iodoacetamide and iodoacetic acid, had a very small inhibiting action. The specificity of the action on thiol groups was confirmed in all cases by reversal of the inhibition with thiol-reducing agents. As shown in Table II, the action of all SH inhibitors was rapidly reversed with 0.005 *M* glutathione or cysteine and normal motility was restored. Simple washing with saline or buffer solution did not reverse the action of the thiol inhibitors while the effect of the reducing agents was in some cases instantaneous.

Action of narcotics and other inhibitors. Agents known to act on the oxidative mechanisms of bacteria, such as potassium cyanide and sodium azide, had no visible action on the motility of the bacteria during the period of observation. Results with malonate and with sodium fluoride were also negative. Substances having a narcotic effect, such as chloral hydrate, sodium barbital and chloroform, inhibited motility at high concentrations. One of the most active seems to be chloroform, while

the least active seems to be barbital. Phenobarbital was more active than barbital and its action was rapidly reversed by washing. Complete inhibition with chloral hydrate required a concentration 300 times higher than with p-chloromercuribenzoate. In addition to this difference the inhibition by these narcotics was restored by simple washing. Metadon (chlorhydrate of 4-4 diphenil 6 dimethyl aminoheptenone) had the strongest effect and, after acting for 4 minutes, its inhibition could not be reversed. The recovery depended, among other factors, on the concentration and on the time of action of the narcotic. In Table III are recorded the periods of time after washing before vibration and translation started. In these cases chloral hydrate 0.3 *M* had previously acted for 60 seconds. Complete recovery of motility took, however, much longer periods. In 2 experiments with phenobarbital the motility was apparently restored more rapidly with amphetamine sulfate (Benzedrine) 0.03 *M* than with buffer.

Action of adenosintriphosphate. In several experiments it was observed that ATP had a definite stimulating effect on the motility of *Proteus vulgaris*. This was particularly clear in some slow forms of this bacillus. However, the action was difficult to detect in rapidly moving bacteria. In order to obtain results which could be observed objectively and expressed numerically the following method was used. Bacteria were paralyzed completely by the action of chloral hydrate (0.3 *M* for 60 sec.) and a record was taken of the time in which vibration and translation started after washing with saline buffer of pH 7 (Table III). Afterwards the same bacteria were treated again with chloral hydrate and the recovery was obtained by washing them with ATP or other phosphate compounds. Since the effect of the second treatment with the narcotic is normally more prolonged than the first, ATP was always applied after the second inhibition. As indicated in Table III, in spite of this handicap ATP induced recovery of vibration and translation in a much shorter time than the buffer, the saline solution, or the phosphate compounds: Sodium pyrophosphate, 3-phosphoglyceric acid, glucose 1-6 phosphate, and adenylic acid (the latter not indicated in the table). Although the times varied considerably from one experiment to another, the differences are clear. With ATP vibration started after a period about 110% shorter than in the controls while translation started after a period 74% shorter. The activating action of ATP was inhibited by a previous treatment with a strong thiol inhibitor such as p-chloromercuribenzoate.

Discussion. The special technic described here may open a new way for obtaining information about the fundamental factors involved in bacterial motility since it makes it possible to observe for any length of time the motility of individual bacteria submitted to the action of different chemical or physical agents. The use of a chamber with permeable wall has many other possibilities since it is possible in this way to study the effect of chemical agents on actively multiplying cells as well as on "resting suspensions." Problems of bacterial growth and multiplication could also be investigated. The use of cellophane membranes in the culture of microorganisms has been already described, but only with the purpose of obtaining high concentrations of bacteria in a small volume(6). This membrane is permeable to drugs of small molecular weight, like those used in this work. Although the rate of diffusion across the membrane was not investigated, the results obtained with narcotics or with —SH reagents (the latter showed activity in about a few seconds) point to a fairly fast action. Negative results with drugs of a similar molecular weight could not possibly be due to lack of diffusion.

The reversal of the inhibitory action of several compounds by simple washing with saline or by the use of specific reducing agents indicates that it is not safe to take inhibition of motility as a direct manifestation of lethal effect. Buchbinder and Zaretsky's test(7) for the determination of the bactericidal concentration of quaternary ammonium compounds cannot therefore be accepted without further

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analysis as a general test for bactericidal action.

Motility of bacteria and SH groups. Experiments on the action of thiol inhibitors indicate that —SH groups are probably involved in the motility of *Proteus vulgaris*. Among those inhibitors the mercaptide-forming agents had the strongest action and could stop the bacterial movement entirely. Of these agents p-chloromercuribenzoic acid has the advantage of combining with single R-SH groups and of being, according to Barron(8), "the most powerful inhibiting agent and the most specific when accompanied by reactivation." The other thiol inhibitors of the oxidizing type and particularly those having an alkylating action such as iodoacetate and iodoacetamide had a smaller effect. Similar differences are often found in —SH systems (8). The action of these inhibitors could not be reversed by simple washing in buffer or saline, while motility was immediately restored with the specific reducing agents of the SH groups: glutathione and cysteine. The action of thiol inhibitors on bacterial motility may probably be interpreted as evidence for the presence of essential —SH groups in the contractile protein of bacterial flagella or in enzyme specially involved in the mechanism of flagellar contraction. In favor of this hypothesis are also the electron microscope observations of De Robertis and Franchi(2) showing that SH inhibitors interfere with the contracting action of ATP on the purified flagellar protein of *Bacillus brevis*. This action is also similar to that previously observed in contractile proteins obtained from muscle. Barron and Singer(9) found that p-chloromercuribenzoate completely inactivated the ATPase activity of myosin while alkylating agents such as iodoacetate and iodoacetamide had no action. Furthermore, Bailey and Perry(10) demonstrated that SH groups are essential for the formation of actomyosin. In view of these facts it would be of interest to study the ATPase activity of bacteria and to see if it is present in purified flagella.

Motility of bacteria and ATP. Nothing is known about the source of energy used by flagella during contraction. The lack of effect on motility by compounds known to have an inhibitory action on the oxidative cycle or on glycolysis might be attributed to the metabolic characteristics of *Proteus vulgaris*. It seems probable that a different effect may be found in bacteria having other mechanisms for obtaining energy. In our experiments the only agent found to have a definite stimulating effect on the motility of *Proteus vulgaris* is adenosintriphosphate. All the other inorganic and organic phosphates employed had no visible action on locomotion. Furthermore, our previous electron microscope observations (2) show that flagellar protein contracts under the action of ATP, but not with other phosphate compounds. Since the discovery of the ATPase activity of myosin(11) and the demonstration that ATP induces contraction on actomyosin(12) the role of this compound on muscle contractility has been extensively investigated. ATP seems to have also an important role in the motility of sperm (Lardy *et col.*)(13). The results reported here show that ATP may be also one of the sources of energy involved in bacterial motility and in the contraction of bacterial flagella.

Conclusions. (1) The motility of *Proteus vulgaris* × 19 has been studied in a special chamber which permits prolonged and undisturbed observation of a group of bacteria under the influence of different chemical agents. (2) Strongest inhibition was observed with thiol inhibitors of the mercaptide-forming type, particularly with p-chloromercuribenzoate. The oxidizing agent iodosobenzoate and particularly the alkylating-SH drugs, iodoacetamide and iodoacetate, had a

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less marked inhibitory action. The specificity of the action of thiol inhibitors was confirmed in all cases by reversal of the inhibition with the thiol-reducing agents: glutathione and cysteine. Potassium cyanide, sodium azide, sodium fluoride and malonate had no action on the motility of *Proteus vulgaris*. (3) Substances having a narcotic effect, such as chloral hydrate, barbital, phenobarbital and chloroform, inhibited motility only at high concentrations. In these cases recovery of motility could be obtained by simply washing in buffer or saline. (4) Adenosintriphosphate had a definite stimulating effect on the motility of *Proteus vulgaris*. It was found that in bacteria inhibited with chloral hydrate vibration and translatory movements started within periods of time which were 110 and 74% shorter with ATP than with saline or other organic or inorganic phosphates. The possible role of —SH groups and of ATP in bacterial locomotion is discussed.

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Size and Shape of Blood Group *A*-Substance. (19149)

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Kekwick has recently presented data on the size and shape of an A-substance preparation from pseudomucinous ovarian cyst fluid(1). He obtained a molecular weight of 260000 and an axial ratio of 60/1 from ultracentrifuge and diffusion measurements. For comparison it seems worthwhile to present briefly data obtained on other preparations of A-substance(2).

Materials. The closely related preparations R18-F10, R19-F1A, and R19-F2A of A-substance from hog gastric mucin were used (3,4).‡ This material is known to be chemically and immunologically inhomogeneous (3,4). The solvent used in all experiments was 0.02 M phosphate buffer at pH 7.1 with sufficient sodium chloride to give an ionic strength of 0.15.

Light scattering. Measurements were made at concentrations between 1.0 and 0.1%. Values of the reciprocal of specific turbidity were plotted against concentration and extrapolated to zero concentration. The molecular weights of R19-F1A and R19-F2A calculated from the values of $(C/\tau)_{C=0}$ were 900,000 and 1,100,000 respectively. All samples were centrifuged for 30 minutes at 25,000 times gravity to remove optical haze. The refractive index increment for 5461 Å was 0.14 (concentration expressed as weight fraction).

Diffusion. Free diffusion measurements at 2 concentrations of R18-F10 were made at 1.3°C in a Neurath cell using a Longsworth scanning optical system. By the height-area method

$$D^{20^\circ}_{H_2O} = 1.12 \pm 0.11 \cdot 10^{-7}\ \text{cm}^2/\text{sec.}$$

and by the method of successive analysis

$$D^{20^\circ}_{H_2O} = 1.03 \pm 0.07 \cdot 10^{-7}\ \text{cm}^2/\text{sec.}$$

for 5 determinations. Pycnometric measurements gave an apparent specific volume of 0.66 ml/g.

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