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THE EFFECT OF ALKYL AND ALKENYL SUCCINIC ACIDS ON CELL SIZE AND METABOLISM OF A STRAIN OF *PSEUDOMONAS AERUGINOSA*

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Abstract—Washed cells of a strain of *Pseudomonas aeruginosa* rapidly decrease in size when placed in salt solutions. Upon incubation a slow increase in size occurs as a result of ion influx. This increase is accelerated in the presence of alkyl and alkenyl succinic acids with chain lengths greater than 10 carbon atoms and is proportional within limits to the concentrations of the detergents. As a result of the ion influx the oxidation of succinate and benzoate is accelerated. Salicylate inhibition of the oxidation of succinate and benzoate is eliminated by the detergents; streptomycin inhibits succinate oxidation only in their presence. The detergents partially reverse streptomycin inhibition of benzoate oxidation.

ANIONIC detergents react with positively charged groups on proteins[1, 2] and change their configurations as indicated by changes in physical–chemical characteristics such as viscosity and reaction to heating. The positive charge is primarily contributed by amino and amide groups, since anionic compounds are more effective in acid solutions.[3] When these groups are saturated by the detergent, nonpolar forces between the alkyl chains may bind more of it. The negative charge of these added molecules is now oriented outward from the protein chains and by electrostatic repulsion further distort the protein configuration.[2] These considerations in general apply to the interaction of anionic drugs with bacteria. Work with protoplasts[4, 5] indicates that the protein of the cell membrane is the primary target of these compounds, since membrane disruption occurs which could result from configurational changes in the proteins. Inorganic cations on the membrane, such as calcium and iron which may contribute to its integrity, could also react with anionic drugs. Iron and to a lesser extent calcium counteract some of the effects of an alkyl succinic acid[6] and calcium increases the amount of dodecyl sulfate bound by both Gram-positive and -negative organisms.[7] On the other hand, cationic drugs are more reactive in alkaline solutions[3] and probably combine with phospholipids.[8, 9] Added phospholipids counteract their effects.[10]

Once changes have occurred in the cell membrane, leakage of intracellular constituents into the medium may occur.[11] Purines, pyrimidines, amino acids, and phosphate have been identified.[12] The effects of certain drugs may be increased[13–15] or they may not be,[16] dependent upon whether their entrance into the cell is facilitated or not. Facilitation of metabolite interchange probably accounts for the increase in respiration and glycolysis, which occurs in certain organisms exposed to relatively low concentrations of detergents[17, 18] and the increase in rate of reduction of methylene blue by yeast exposed to alkyl succinic acid half esters.[19]

The following is a study of the effects of alkyl and alkenyl succinic acids on a strain of *Pseudomonas aeruginosa*. This organism and many other Gram-negative ones decrease in size when exposed to solutions of electrolytes or nonelectrolytes.[20] The factors effecting this change have been studied in some detail.[21] When washed cells of *P. aeruginosa* are put into salt solutions an immediate decrease in cell size occurs, which reaches a maximum within 60 sec. Upon incubation the cells gradually swell as the result of the uptake of ions and their water of hydration. This conclusion is based on the fact that osmolar sucrose solutions decrease cell size to the same extent, but subsequently little or no swelling occurs unless an inorganic salt is added. It was thus possible to study the change these anionic compounds cause on ion uptake in this organism and the consequences of the change on certain metabolic activities and the effect of drugs.

EXPERIMENTAL

A strain of *P. aeruginosa* maintained in this laboratory for 15 years was grown at 34° for 24 hr in 100 ml Difco nutrient broth without shaking. The cells were centrifuged down and washed twice with distilled water. They were then immediately suspended in 0·05 M Na–K phosphate buffer, pH 6·7. At pH 6·0 most substrates were not oxidized; at pH 7·4 the detergents were inactive. The density of the suspension after the addition of the buffer was adjusted to a standard value by measuring light absorption in a Coleman Jr. spectrophotometer at 500 mμ. This instrument was also used to measure density changes which occurred on incubation with and without the addition of the detergents. It has been shown that under the experimental conditions optical density changes measure change in cell size and density.[20, 21] The optical density (O.D.) is expressed as $-\log T \times 1{,}000$. The effect of the detergents on the oxidation of acetate, succinate, and benzoate was determined by the standard Warburg method. Unpublished work with this organism has shown that acetate oxidation proceeds without a lag period, which indicates that it requires no transport mechanism or is oxidized at the cell membrane. Succinate oxidation shows a lag period indicating the induction of a permease, and benzoate oxidation shows a longer lag period because enzyme induction is involved. The alkyl and alkenyl succinic acids were kindly donated by the Humphrey Chemical Co., North Haven, Conn. They were free of inorganic salts, and their relative solubilities were consistent with their indicated chain length and saturation.

RESULTS

Alkyl and alkenyl succinic acids with carbon chains of 8, 10, 12, 14, and 16 were used. They were dissolved in water by heating and brought to pH 6·7 by addition of NaOH. The concentrations for maximal effects on oxidation ranged from 500 μg/ml for the 8-carbon to 25 μg/ml for the 14-carbon compounds. The insolubility of the 16-carbon compounds made it difficult to obtain reliable concentration effects. At these concentrations the 8- and 10-carbon compounds incubated with cells had little effect on the rate of increase in cell size (decrease in O.D.) but beginning with the 12-carbon compounds the rate of increase was accelerated. Figure 1 shows the effect of different concentrations of tetradecenyl succinic acid. The O.D. change was roughly proportional to the concentration.

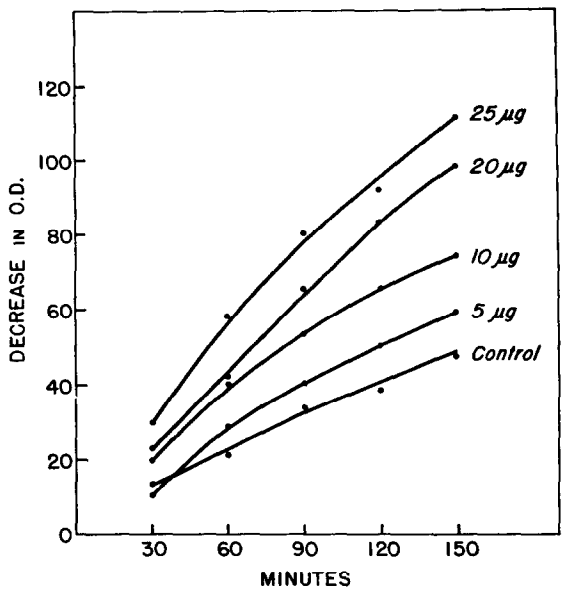

FIG. 1. The decrease in O.D. in the presence of different concentrations of tetradecenyl succinic acid (μg/ml) plotted against time of incubation (37°, pH 6·7, 0·05 M Na–K phosphate buffer).

Table 1 shows the effect of the dodecenyl compound on the O.D. changes in the presence of 0·1, 0·05, and 0·025 M Na–K buffer. The initial O.D. was greater, the higher the buffer concentration. The rate of O.D. decrease followed the same order because, although ion assimilation depends on metabolic activity in this organism,[21]

TABLE 1. THE EFFECT OF DIFFERENT BUFFER CONCENTRATIONS ON THE CHANGE OF O.D. WITH TIME IN THE PRESENCE AND ABSENCE OF DODECENYL SUCCINIC ACID (250 μG/ML)

The initial O.D. values were reached within 60 sec of adding the buffers (37°, pH 6·7).

Buffer conc.	Optical density				Differences		
	0	30′	60′	90′	30′	60′	90′
0·1 M	341	270	240	231	71	101	110
+ dodecenyl	359	172	148	130	187	211	229
0·05 M	282	254	238	230	28	44	52
+ dodecenyl	300	232	198	180	68	102	120
0·025 M	257	248	241	235	9	16	22
+ dodecenyl	259	248	231	224	11	28	35

the diffusion gradient aids this process. At the end of 60 min the O.D. values were essentially the same, apparently because the cell can control its intracellular ion concentration. This was not true in the presence of the detergent. The O.D. values were lower the higher the buffer concentration, which indicates that diffusion whether active or passive can no longer be controlled.

All compounds at whatever concentrations used inhibited the oxidation of acetate. Their effects on the oxidation of succinate and benzoate were as follows. The 8- and 10-carbon compounds which had little effect on cell size inhibited the oxidation. The other compounds accelerated the oxidation 200–400 per cent under optimal conditions. The relative effectiveness of the alkyl and alkenyl series was as follows: octyl > octenyl; decyl = decenyl; dodecyl > dodecenyl; tetradecenyl > tetradecyl; hexadecenyl > hexadecyl. The reason why the octyl and dedecyl compounds were more effective than their alkenyl analogs is not clear. The greater effectiveness of the tetra and hexadecenyl compounds compared to their alkyl analogs is probably the result of their greater solubility, i.e. a lesser tendency to form micelles. Both 16-carbon compounds required a longer time than the more soluble ones to accelerate the oxidation. Despite the acceleration of the oxidation rate, the lag phase was still present. This might have been due to the slow action of the compounds. Consequently, their effect was compared when they were added with the succinate and 90 min before succinate. Under this condition the lag phase was reduced but not eliminated. Higher concentrations of the detergents could not be used, as lysis occurred and oxidation rates fell off sharply. The lag phase in the oxidation of benzoate was also reduced by

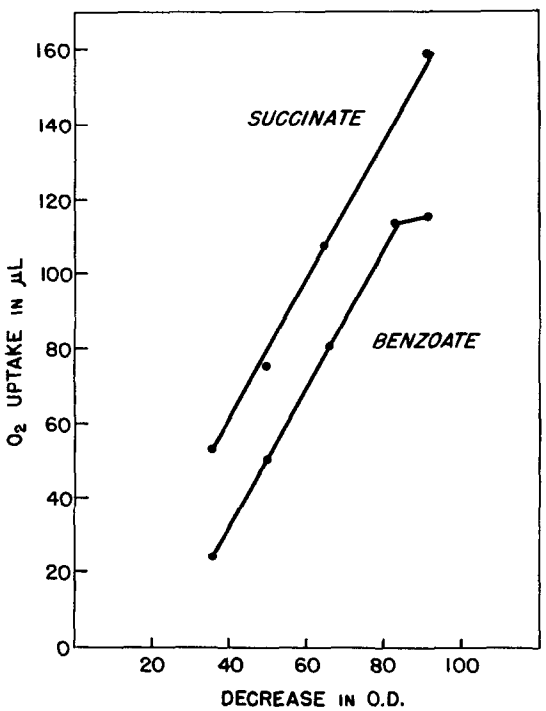

FIG. 2. The O_2 uptake at 120 min in the presence of 0·5 mg Na succinate $6H_2O$/ml or 0·25 mg Na benzoate/ml plotted against the change in O.D. after 120-min incubation (37°, pH 6·7, 0·05 M Na–K phosphate buffer).

preincubation of the detergents with the cells, but in this case complete elimination could not be expected because enzyme synthesis is involved.

Figure 2 shows that if the oxygen uptake, after the lag phase, is plotted against the change in O.D. which had occurred at the same time, in this case after 120-min incubation, a straight line is obtained. This holds for both succinate and benzoate

except that, for the latter, the oxidation rate fails to increase after the cell has reached a certain size. This implies a disruption of intracellular organization which affects enzyme induction before it affects succinate oxidation. Greater changes in O.D., as mentioned above, inhibit both oxidations. Cell size per se is not, however, the determining factor. If, in the absence of the detergent, Na phosphate buffer was substituted for Na–K buffer, the oxidation rate of succinate decreased 60 per cent; if K buffer was substituted, the decrease was 90 per cent. Yet the O.D. change was 40 and 46 units for Na and K, respectively, and 73 units for Na–K buffer. In the same experiment 250 μg dodecenyl compound/ml caused an O.D. change of 88, 133, and 135 units in Na, K, and Na–K buffer, and the corresponding oxygen uptakes were 104, 46, and 153 μliters. Thus there is no correlation between cell size and oxidation rate unless both cations are present. The same relationships hold for benzoate oxidation. The importance of cations is shown by the fact that no oxidation occurred when none was added and the cells were suspended in Tris buffer. Detergents added under these conditions allowed for such a rapid and uncontrolled entry of the Tris ions that lysis occurred within a few minutes.

Salicylate inhibited the oxidation of succinate and benzoate; 30 μg/ml inhibited 50 and 25 per cent respectively. In the presence of a concentration of detergent which increased oxidation rate the inhibition was greatly reduced. This is shown in Table 2 for succinate. Salicylate did not affect the rate of swelling either in the presence or absence of the detergent. *m*-Hydroxybenzoate behaved similarly, and therefore chelation of metal ions cannot account for the salicylate inhibition.

Streptomycin sulfate (50 μg/ml) had no effect on the oxidation of succinate, but in the presence of the detergent marked inhibition occurred (Table 2). Since streptomycin is a cation, it might form a salt with the anionic detergent and thus counteract the acceleration of oxidation by the latter. That this is not the explanation is shown by the following facts. (1) Streptomycin had no effect on swelling caused by different concentrations of detergent. (2) An increase in detergent concentration increased the inhibition of oxidation. (3) The inhibition occurred in the presence of 250 μg dodecenyl compound/ml to the same extent as with 25 μg tetradecenyl compound/ml. (4) The oxygen uptake was well below that of the control. Thus the oxidation of succinate is inhibited if streptomycin has access to the enzyme. Umbreit[22] has shown that streptomycin inhibits the pyruvate–oxalacetate reaction in *Escherichia coli* and thus indirectly the oxidation of succinate.

Streptomycin inhibited benzoate oxidation because enzyme synthesis is involved, as first shown in mycobacteria.[23] The inhibition was much less in the presence of the detergent so that oxidation rate approximated that of the control (Table 3). Again, different detergent concentrations had little influence on this effect and, in the presence of the octenyl compound in a concentration inhibiting benzoate oxidation slightly, the inhibition by streptomycin was unaffected. Since the octenyl compound should make a salt with streptomycin if the other compounds do, salt formation cannot account for the inhibition reversal.

DISCUSSION

The alkyl and alkenyl succinates when used in proper concentrations cause a slow increase in cell size as a result of ion influx, and this made it possible to measure certain enzyme activities under different intracellular ion concentrations. The changes

TABLE 2. THE EFFECT OF 30 μG NA SALICYLATE/ML AND 50 μG STREPTOMYCIN SULFATE/ML ON THE OXIDATION OF 0·5 MG NA SUCCINATE $6H_2O$ IN THE PRESENCE AND ABSENCE OF TWO CONCENTRATIONS OF DODECENYL SUCCINIC ACID

The figures are in microliters O_2 uptake. The drugs and detergent were added 10 min before the succinate. The small autorespiration which was not affected by the detergent has been subtracted (37°, ph 6·7, 0·05 M Na–K phospate buffer).

Min	Succinate	+ Sal.	Inhibition %	+ Strept.	Inhibition %	+ 150 μg Dodecenyl/ml	+ Sal.	Inhibition %	+ Strept.	Inhibition %	+ 250 μg Dodecenyl/ml	+ Sal.	Inhibition %	+ Strept.	Inhibition %
60	26	19	27	28	0	29	29	0	24	19	46	42	9	21	54
100	53	32	40	55	0	75	62	17	48	36	114	95	17	37	68
120	74	39	47	76	0	119	100	16	60	50	158	121	23	42	73
150	120	52	57	126	0	172	152	12	72	58	178	154	13	46	74

TABLE 3. THE EFFECT OF DODECENYL AND OCTENYL SUCCINIC ACIDS ON THE OXIDATION OF 0·25 mg Na BENZOATE/ML IN THE PRESENCE AND ABSENCE OF 25 μG STREPTOMYCIN SULFATE/ML

The figures are in microliters O_2 uptake. The detergents and streptomycin were added 10 min before the benzoate. The small autorespiration which was not affected by the detergent has been subtracted (37°, pH 6·7, 0·05 M Na–K phosphate buffer).

Min	Benzoate	+Strept.	+125 μg Dodecenyl/ml	+125 μg Dodecenyl/ml +strept.	+250 μg Dodecenyl/ml	+250 μg Dodecenyl/ml +strept.	+250 μg Octenyl/ml	+250 μg Octenyl/ml +strept.
30	0	0	0	0	2	0	0	0
60	0	0	6	2	17	7	0	0
90	5	0	36	12	49	18	5	0
120	25	0	97	36	110	32	20	0
150	65	0	167	66	166	57	47	0

produced in the membrane by these agents must be complex. The size increase begins abruptly when the 12-carbon compounds are used (the 8 and 10 being relatively inactive at comparable concentrations), and the effectiveness increases, as might be expected, with the chain length until the compounds become too insoluble. All the compounds inhibit the oxidation of acetate, a substrate that exhibits no lag period and therefore requires no transport mechanism or is oxidized on the membrane. Either entrance of the substrates into the cell or access to the enzyme is inhibited.

Since the oxidation of succinate and benzoate does not occur in the absence of cations, and increase in cell size does not occur in their absence, the obvious explanation for the acceleration is the greater concentration of intracellular cations in cells exposed to the detergents. In the normal cell, cation assimilation is dependent on energy. In the detergent-treated cell, cations may enter in large part by passive diffusion. Thus in the normal cell the oxidation rate of certain substrates may initially be slow but, if cations are being assimilated, the rate would increase with time and a sigmoid curve obtained. This effect might explain some lag periods.

The use of certain detergents may be useful in localizing drug action. In *P. aeruginosa*, the effect of salicylate is apparently only on the membrane. The effects of streptomycin seem to be complex. Its penetration into the normal cell is indicated by the fact that the enzyme induction is inhibited. Yet succinate oxidation is not inhibited unless a detergent is present. The detergent, therefore, must allow access of the drug to the enzyme, which it does not have in the normal cell. The reversal of streptomycin inhibition of benzoate enzyme induction by the detergent may be the result of ion accumulation which may interfere with the action of the antibiotic on enzyme synthesis.

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