Similar arguments might be invoked to explain the results obtained with isotetracycline. That is to say, these alterations may alter the pKa spectrum of these derivatives, adversely affecting their transport to the bacterial enzyme site.

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Mechanism of Action of Phenolic Disinfectants VI

Effects on Glucose and Succinate Metabolism of *Escherichia coli*

By HENRY COMMAGER and JOSEPH JUDIS

A representative group of phenols was examined as to effects on aerobic and anaerobic utilization of glucose and aerobic utilization of sodium succinate. At low concentrations, *p*-chlorophenol, 2,4-dichlorophenol, *p*-chloro-*o*-cresol, and 2,4-dinitrophenol stimulated oxygen uptake with glucose as the substrate; phenol and *p*-chloro-*m*-xylenol were inhibitory, and *p-tert*-amylphenol had no measurable effect. At the higher concentrations used, all compounds except 2,4-dinitrophenol were inhibitory. Anaerobic metabolism of glucose was inhibited by concentrations of the compounds tested similar to those inhibiting glucose oxidation. Oxidation of sodium succinate was stimulated by low concentrations of *p*-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, *p*-chloro-*o*-cresol, dichloro-*m*-xylenol, and *p-tert*-amylphenol. All compounds were inhibitory at higher concentrations. In general, sodium succinate oxidation was the most sensitive to phenolics, and glucose oxidation the least sensitive. There was some correlation between phenol coefficients and inhibition of glucose and succinate metabolism. It is hypothesized that the lethal action of phenolic disinfectants is due to damage of permeability mechanisms, the repair of which is prevented by concomitant inhibition of energy-yielding metabolic reactions.

PREVIOUS WORK from this laboratory (1–3) strongly suggests that phenol disinfectants caused damage to permeability mechanisms in bacteria, though definitive correlation between such damage and the lethal effects of phenolic disinfectants must yet be established. Permeability damage would probably have to be rather extensive and irreversible for loss of macromolecules, such as proteins or nucleic acids, but the type of damage detected by β-galactosidase activity in cryptic (permeaseless) strains of

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TABLE I.—EFFECT OF PHENOL DISINFECTANTS ON THE UTILIZATION OF GLUCOSE OR SODIUM SUCCINATE BY *E. coli*

Phenol Derivative	Concn. of Phenol Derivative in Reaction Flask which Caused 50% Inhibition[a] (MIC 50%)					
	Aerobic Glucose Utilization,		Anaerobic Glucose Utilization,		Aerobic Sodium Succinate Utilization,	
	mcg./ml.	μM/ml.	mcg./ml.	μM/ml.	mcg./ml.	μM/ml.
Phenol	3050	32.4	3700	39.3	1100	11.8
p-Chlorophenol	860	6.7	590	4.6	690	5.4
2,4-Dichlorophenol	290	1.8	200	1.3	37	0.28
2,4,6-Trichlorophenol	...	...	164	0.82	94	0.47
p-Chloro-*o*-cresol	390	2.7	...	...	117	0.82
p-Chloro-*m*-xylenol	180	1.2	105	0.67	...	...
Dichloro-*m*-xylenol	65	0.35	64	0.33	54	0.28
2,4-Dinitrophenol	[b]	[b]	190	1.0	...	...
p-tert-Amylphenol	161	0.98	...	...	73	0.44

[a] Determined graphically on log probability paper. [b] Stimulated oxygen uptake at all concentrations tested.

Escherichia coli would be very slight since entry of a relatively small molecule is involved. A number of phenol derivatives have been shown to cause the latter type of damage as well as more extensive damage, depending on concentration (4). It is conceivable that cells whose membranes were affected to a minor degree could repair the disruptions and recover, if energy from metabolic reactions were available and if phenol disinfectants did not also concomitantly inhibit energy-yielding reactions. To determine whether such inhibition, *i.e.*, of energy yielding reactions, is caused by phenol disinfectants, the effects of several of the latter on both aerobic and anaerybic glucose metabolism and oxidation of succinate were surveyed.

METHODS AND MATERIALS

Bacteria.—*E. coli* ATCC 11229 was used throughout and was maintained on nutrient agar. Growth conditions and preparation of cell suspensions were as reported previously (3). Culture media contnined either 0.5% glucose or sodium succinate, depending on whether glucose or succinate metabolism, respectively, was being studied. Cell suspensions had a concentration of 40 mg. wet weight per ml., corresponding to 10 mg. dry weight per ml. or 4×10^{10} cells per ml.

Manometric Procedures.—Oxygen uptake and carbon dioxide release were measured in a Warburg respirometer using standard techniques (5). For measurement of oxygen uptake, the flasks were prepared as follows. In the center well, 0.2 ml. of a 20% solution (w/v) of potassium hydroxide was placed with a small strip of filter paper. One side arm contained 0.4 ml. of a 1% (w/v) solution of glucose, or if sodium succinate was the carbon source, 0.4 ml. of a 1% (w/v) solution of sodium succinate. The other side arm contained the test chemical which was dissolved in 0.1% sodium hydroxide and sufficient 0.1% sodium hydroxide to bring the total volume of the solution in the sidearm to 0.4 ml. The reaction chamber contained 0.9 ml. of 0.15 *M* potassium phosphate buffer, pH of 6.8, 0.6 ml. of the cell suspension described above, and sufficient distilled water to bring the total volume of solution to 3.0 ml. when the test substances were tipped in. The final pH of the reaction mixture was 7.2, and the gas phase was air.

For the measurement of carbon dioxide production (anaerobic metabolism of glucose), the flasks were prepared as follows. In one side arm 0.2 ml. of a 0.04 *M* solution of glucose was placed, and in the other side arm, the test substance dissolved in water (depending on its solubility) or in 25% (v/v) ethanol. It was found in preliminary experiments that glucose utilization under anaerobic conditions was not measurably affected by the presence of ethanol in the concentrations used. In the other side arm of the controls, 0.4 ml. of 25% ethanol was placed. The reaction chamber contained 2.0 ml. of 0.02 *M* sodium bicarbonate, 0.6 ml. of cell suspension, and sufficient distilled water to bring the total volume of liquid to 3.2 ml. After the flasks were prepared, they were attached to the manometers and gassed for 20 min. with a 95% nitrogen–5% carbon dioxide mixture. Both side arms were tipped at the same time, and the temperature was maintained at 30° for all manometric experiments. The shaking rate was 110 strokes/min.

The determination of the median inhibitory concentration 50% (MIC 50) was done graphically using Keuffel and Esser log probability paper No. 358-22 in the manner described by Finney (6).

The phenol derivatives were obtained from Distillation Products Industries with the exception of *p*-chloro-*m*-xylenol, *p*-chloro-*m*-cresol, and dichloro-*m*-xylenol, which were a gift of the Ottawa Chemical Co., Toledo, Ohio. All other chemicals used were of reagent grade.

RESULTS AND DISCUSSION

A number of concentrations of each of the phenol derivatives was used representing very low concentrations which were known to have no germicidal effects as well as higher concentrations shown to be lethal to a high proportion of the cells, generally 99% killing (1, 7). Table I presents a summary of the results. The 50% inhibitory concentration was determined graphically on log probability paper. From the data obtained, it appears that sodium succinate oxidation was more sensitive than either aerobic or anaerobic utilization of glucose and that as phenol is halogenated and/or alkylated, inhibition of glucose or sodium succinate utilization is intensified.

There has been a great deal of study on the effects

TABLE II.—EFFECTS OF LOW CONCENTRATIONS OF PHENOL DERIVATIVES ON GLUCOSE AND SUCCINATE OXIDATION

Phenol Derivative	Glucose Oxidation: Concn. Causing Max. Stimulation, mcg./ml.	Glucose Oxidation: Lowest Concn. Tested, mcg./ml.	Glucose Oxidation: % of Control	Succinate Oxidation: Concn. Causing Max. Stimulation, mcg./ml.	Succinate Oxidation: Lowest Concn. Tested, mcg./ml.	Succinate Oxidation: % of Control
Phenol	...	1670	78	333	333	115
p-Chlorophenol	166	166	113	133	133	119
2,4-Dichlorophenol	16.7	16.7	126	16.7	16.7	118
2,4,6-Trichlorophenol	...	...	...	33.3	33.3	112
p-Chloro-*o*-cresol	66.7	33.3	133	33.3	33.3	141
Dichloro-*m*-xylenol	...	24.8	100	16.7	16.7	112
p-*tert*-Amylphenol	...	33.3	100	8.4	8.4	120
2,4-Dinitrophenol	37.7	7.37	147	...	...	...

TABLE III.—RELATIONSHIPS OF METABOLIC INHIBITORY CONCENTRATIONS OF PHENOLIC DISINFECTANTS TO PHENOL COEFFICIENTS AND WATER SOLUBILITIES

Phenol Derivative	Phenol Coefficient[a]	Solubility in Water, Gm./100 ml.[b]	MIC-50%, μM./ml.: Glucose Oxidation	MIC-50%, μM./ml.: Succinate Oxidation
Phenol	1.0	6.7[g]	32.4	11.8
p-Chlorophenol	3.9–4.3	2.71[h]	6.7	5.4
p-Chloro-*o*-cresol	10.7–12.5	[c]	2.7	0.82
2,4-Dichlorophenol	13	0.46[h]	1.8	0.28
p-Chloro-*m*-xylenol	30–36	0.03[h]	1.2	...
2,4-Dinitrophenol	Less than 1	0.56	1.0[d]	...
p-*tert*-Amylphenol	30	[c]	0.98	0.44
2,4,6-Trichlorophenol	23–40	0.08[h]	0.82[e]	0.47
Dichloro-*m*-xylenol	250[f]	[c]	0.35	0.28

[a] Phenol coefficients were obtained from Reddish (17) or Sykes (18). [b] Solubilities were obtained from Heilbrun (15) or *Reference 29*. [c] Value not available in the literature. [d] All concentrations tested against glucose oxidation were stimulatory, and therefore MIC-50% for anaerobic utilization of glucose is given. [e] MIC-50% for anaerobic glucose utilization. [f] Rideal-Walker coefficient (18). [g] 16°C. [h] 20°C.

of 2,4-dinitrophenol on metabolism of both mammalian systems and bacteria (8–10). It has been well established that this compound interferes with phosphorylation leading to the production of ATP. Gaur and Beevers (11) extended such studies to other phenolic compounds to determine whether derivatives of phenol besides 2,4-dinitrophenol could act as uncouplers. These workers used carrot disks as experimental material and found that oxygen uptake was indeed stimulated by certain concentrations of a number of halo- and alkylphenol derivatives. Hugo (12), in studies on the effects of phenol and 2-phenoxyethanol on *E. coli*, also observed stimulation of oxygen uptake with mannitol, glucose, and lactose as substrates. It seemed of interest to survey in a preliminary fashion for similar effects with the phenol derivatives used in this study. The results are illustrated in Table II. While the stimulatory effects obtained were not as dramatic as seen with 2,4-dinitrophenol in the mammalian or carrot systems, oxygen uptake with glucose or sodium succinate as substrate was significantly and consistently stimulated beyond that of the control and may represent uncoupling. Certainly further work would be necessary to establish the conclusion that true uncoupling was obtained.

From Table II it can be seen that at low concentrations, *p*-chlorophenol, 2,4-dichlorophenol, *p*-chloro-*o*-cresol, and 2,4-dinitrophenol stimulated oxygen uptake with glucose as the substrate; phenol and *p*-chloro-*m*-xylenol were inhibitory, and *p*-*tert*-amylphenol had no measurable effect. Oxidation of sodium succinate was stimulated by low concentrations of *p*-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, dichloro-*m*-xylenol, and *p*-*tert*-amylphenol.

Any deleterious effect which can be shown to occur when bacteria are exposed to phenolic disinfectants should, presumably, be relatable to the potency of the individual phenol disinfectants. That is, as the intensity of the deleterious effect increases for a series of phenol disinfectants, the phenol coefficient of these derivatives should increase in the same direction. Furthermore, with increasing halogenation and/or alkylation, phenol becomes more germicidal; it also becomes more water insoluble and more lipid soluble. It has been shown in previous work from this laboratory that binding of radioactive phenol derivatives to bacteria is greater with the decreasing water solubility of the derivative and increasing germicidal potency (3, 7).

From Table III it can be seen that the MIC 50% values for glucose and succinate oxidation decreased as the phenol coefficients increased and water solubilities decreased. At the pH of the reaction mixtures (7.2 throughout), the phenol derivatives were primarily in the unionized form with the exception of 2,4,6-trichlorophenol, which has a pK of about 7.5, and 2,4-dinitrophenol with a pK of 4.0 (14, 15). The pK of 2,4-dichlorophenol is 7.8, and the other derivatives have higher pK values (16). In the case of phenol derivatives with pK's less than about 8.5, one is measuring effects of a mixture of free phenol and phenate, and since the latter may be less active than the free phenol (17, 18), one is dealing with minimal effects where a significant amount of phenate is present. It would

appear that the germicidal potency of a given phenol derivative may not necessarily be inherent in the molecule itself but is possibly related to how much of the substance becomes associated with the bacterial cells. There is undoubtedly a partitioning that occurs between the aqueous medium in which the bacteria are suspended and the bacteria for the phenol derivative, and the less water soluble a derivative is the more it will tend to associate with the lipids of the bacterial cells. It is of interest to note that halo- or alkyl- derivatives of phenol which are much less water soluble and much more lipid soluble than phenol were also more potent than the latter in causing release of radioactivity from *E. coli* labeled by growth on various radioactive substrates (2). The same picture was obtained in studies on ability of phenol derivatives to break the permeability barrier to β-galactosidase substrate for a cryptic (permeaseless) strain of *E. coli* (4). In a study of uncoupling effects of phenols, Hemker (13) noted that lipid solubility was an important factor.

The somewhat greater sensitivity of succinate oxidation to phenol derivatives is an expected one if one concludes that phenol derivatives have their primary effects on the cell membrane because succinic dehydrogenase is known to be associated with the cell membrane in *E. coli* (19–23). In previous work, it was found that incorporation of label from ^{14}C succinate into growing cells of *E. coli* was markedly inhibited at concentrations of 2,4-dichlorophenol not affecting incorporation of radioactivity from other substrates (24).

In conclusion, it appears that low concentrations of certain phenolic germicides stimulated oxidation of glucose and sodium succinate, while higher concentrations approaching those shown to be lethal for *E. coli* were definitely inhibitory to aerobic and anaerobic metabolism of glucose as well as aerobic metabolism of sodium succinate. There was a definite relationship between the MIC 50% for the phenol derivatives tested and phenol coefficients. These findings correlate well with previous experimental results which indicated a relationship between ability to damage the cell membrane of *E. coli* and the phenol coefficient. Of course, it is not possible to state in which order these effects occur, that is, whether the membrane is first damaged leading to inhibition of the metabolic reactions tested or whether metabolism is first disturbed with later damage to the cell membrane. The concentrations causing cell membrane damage in *E. coli* ML 35 (4) and the MIC 50% values for aerobic glucose utilization as reported in Table I above are relatively close, although the MIC 50% values for aerobic succinate utilization are lower than those observed for cell membrane damage in *E. coli* and thus may indicate that the succinate utilizing system is more sensitive to phenol derivatives than integrity of the cell membrane. In this connection, Sykes (25) found that the concentrations of a number of germicides causing inhibition of succinic dehydrogenase of *E. coli* were less than the lethal concentrations. If a trend exists, it seems to be that glucose and succinate metabolism are inhibited by lower concentrations of phenol derivatives than those causing cell membrane destruction and killing, and one might therefore conclude that this inhibition of metabolism makes it impossible for the cell to repair the damage caused by cell membrane disruption, thus leading to death of the cell. It may also explain why germicide-treated cells show higher survival rates when plated on certain media, possibly because conditions are provided for metabolic activity leading to repair of membrane damage (26, 27). In this regard, Bean and Walters (28) showed an increased survival rate with bacteria treated with a phenolic germicide if cell exudate from damaged cells was present. Apparently, this exudate served as a nutrient which may have facilitated cell repair.

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