Mechanism of Action of Phenolic Disinfectants V

Effect of 2,4-Dichlorophenol on the Incorporation of Labeled Substrates by *Escherichia coli*

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Escherichia coli ATCC 11229, when exposed to concentrations of 2,4-dichlorophenol adequate to inhibit growth in synthetic medium, was able to incorporate some label from glucose-C-14 and sodium sulfate-S-35 but little from thymine-C-14, acetate-C-14, or succinate-C-14. This growth inhibiting concentration of 2,4-dichlorophenol (66.5 mcg./ml.) permitted substantial incorporation of radioactivity from adenine-C-14, uracil-C-14, or leucine-C-14, although higher concentrations (133 mcg./ml.) were inhibitory. If incorporation of leucine-C-14 and uracil-C-14 can be taken as indicators of protein and ribonucleic acid synthesis, respectively, the results suggest that a concentration of this germicide adequate to prevent growth does not inhibit synthesis of protein or ribonucleic acid. Also, if one assumes inhibition of label incorporation from radioactive succinate to be due to inhibition of succinic dehydrogenase, known to be located in the cell membrane, damage by the phenol to biochemical mechanisms of the cell membrane would appear to be indicated.

ALTHOUGH THERE is substantial literature on the practical aspects of the use of phenolic germicides (1–4) and some studies on the mechanism of action of these compounds implicating cell membrane damage as a primary effect (5–10), there have been few reports on effects of these agents on microbial metabolism. Sykes (11) noted the inhibition of succinic dehydrogenase of *Escherichia coli* by a number of germicidal phenols, and Gould *et al.* (12, 13) studied the effects of bisphenols on a number of enzymes. Recent work has involved studies on the effects of phenolic germicides on induction and activity of the enzyme, β-galactosidase in *E. coli* (14). Both synthesis and activity of this enzyme were found to be inhibited by the phenols studied, although the former process was more sensitive. Also, the effects of a number of phenolic germicides on metabolism of glucose and succinate were surveryed (15). It was found that oxidation of succinate was inhibited by lower concentrations of the germicides than either oxidation or anaerobic utilization of glucose.

The present study describes the effects of 2,4-dichlorophenol on a number of biosynthetic processes measured by incorporation of radioactivity from certain labeled substrates into cell material.

METHODS AND MATERIALS

Bacteria.—*E. coli* ATCC 11229 was used throughout and maintained on nutrient agar slants.

Experimental Procedures.—Medium C of Roberts *et al.* (16), a synthetic medium, was used in all of the experiments. Glucose, or sodium succinate, separately sterilized in a 50% w/v solution, was added at the time of inoculation to give a final medium concentration of 5 mg./ml. The latter compound was used in the medium only when cells were grown for studies on incorporation of succinate-C-14. Growth flasks which contained 200 ml. of medium in 500-ml. conical flasks were shaken at 37°, the cells harvested by centrifugation, and resuspended in fresh medium to an absorbance of 0.5 ± 0.05 (0.21 ± 0.03 mg. dry weight per milliliter) measured in Bellco nephelo-culture flasks, No. 516, 14 × 130 mm. side arm, in a Bausch & Lomb Spectronic 20 spectrophotometer at 620 mμ. The experimental cultures for determination of incorporation of labeled substrates in the presence of 2,4-dichlorophenol were contained in Bellco nephelo-culture flasks. The total volume of culture was 40 ml., which contained the 2,4-dichlorophenol dissolved in a total volume of 2.66 ml. of a 0.1% sodium hydroxide, labeled substrate in aqueous solution in a total volume of 0.15 ml., 0.4 ml. of glucose or succinate solution, and cells suspended in culture medium to volume. When glucose was omitted, it was replaced by distilled water; the final pH was 7.0–7.2. These cultures were incubated at 30° on a shaker for 210 min., an aliquot centrifuged at the end of the incubation period, the cells washed with distilled water, and resuspended on the basis of absorbance measurements to a concentration of approximately 2 mg. dry weight per milliliter of suspension. Aliquots of the suspensions were dried on concentric ring stainless steel planchets, $^1/_8$ by $1^1/_4$ in., for determination of radioactivity which was assayed with a Nuclear Chicago D47 ultra-thin window flow counter. Sufficient counts were recorded to assure a nine-tenths error below 5%.

Chemicals.—The 2,4-dichlorophenol was obtained from Distillation Products Industries and used without further purification. Sulfate-S-35 was purchased from Abbott Radiopharmaceuticals as sodium sulfate; glucose uniformly labeled-C-14,

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TABLE I.—EFFECT OF 2,4-DICHLOROPHENOL ON INCORPORATION OF THYMINE-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance 0 Time	Absorbance 210 min.
0	0	0.225	87.3	0.45	0.45
5	0	0.56	4689	0.44	0.77
5	66.5	0.26	129	0.42	0.51
5	133	0.24	90	0.43	0.44

[a] A total of 282,000 c.p.m. was added to each culture.

TABLE II.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM ACETATE-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance 0 Time	Absorbance 210 min.
0	0	0.19	11,666	0.44	0.45
0	66.5	0.21	161	0.44	0.41
0	133	0.20	185	0.44	0.43
0	266	0.23	224	0.46	0.41
5	0	0.44	55,469	0.44	0.91
5	66.5	0.18	1,663	0.44	0.46
5	133	0.19	663	0.44	0.38
5	226	0.20	242	0.45	0.38

[a] A total of 184,000 c.p.m. was added to each culture.

TABLE III.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM SUCCINATE-C-14 BY *E. coli*

Sodium Succinate Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance 0 Time	Absorbance 210 min.
0	0	0.21	52,621	0.45	0.45
0	66.5	0.23	3,435	0.47	0.46
0	133	0.24	2,206	0.46	0.47
0	266	0.20	359	0.46	0.44
5	0	0.43	38,016	0.46	0.69
5	66.5	0.20	94	0.46	0.46
5	133	0.22	142	0.46	0.47
5	266	0.19	187	0.46	0.44

[a] A total of 221,175 c.p.m. was added to each culture.

specific activity of 11.4 mc./mmole, was a product of New England Nuclear Corp. The other labeled compounds were supplied by the California Corporation for Biochemical Research and had the following specific activities (millicuries per mmole): thymine-2-C-14, 14.8; sodium acetate-1,2-C-14, 10.5; succinic acid-2,3-C-14, 8.75; adenine-8-C-14, 22.9; uracil-2-C-14, 22.9; and DL-leucine-1-C-14, 4.03. The radioisotopes were dissolved in distilled water to an activity of approximately 2 μc./ml. before use. No unlabeled carrier was added; thus, only trace amounts of labeled compounds were involved, except in the cases of succinate and glucose, where the latter compounds were present as energy sources.

RESULTS AND DISCUSSION

The approach used in this work represents an attempt to survey a number of biochemical processes with the hope of identifying a pattern suggesting a specific site of inhibition by 2,4-dichlorophenol. A concentration of 66.5 mcg./ml. of the latter phenol represented approximately the minimum inhibitory concentration for growth under the experimental conditions. The results seem to indicate that the effects of the phenol at this concentration completely suppressed incorporation of some substrates, partially suppressed others, and slightly suppressed in the remainder. Examples of marked suppression of labeled compound incorporation can be seen in the cases of thymine-C-14, acetate-C-14, and succinate-C-14 as substrates (Tables I–III). Partial inhibition of labeling was found with glucose-C-14 and sodium sulfate-S-35 (Tables IV and V). Least inhibition of labeling was found in the cases of adenine-C-14, uracil-C-14 and leucine-C-14 (Tables VI–VIII). In the latter compounds, the cells incorporated significant label at a concentration of 133 mcg./ml. of 2,4-dichlorophenol.

Analysis of the results requires knowledge of the synthetic mechanisms involved in the incorporation of each of the labeled substrates. Roberts *et al.* (16) in their extensive studies on biosynthesis in *E. coli* examined the distribution of label in major cell components of *E. coli* grown on various labeled substrates. They found, for example, that label from glucose-C-14 was generally distributed between cell protein, nucleic acids, and lipids, with the greatest proportion in proteins. Acetate-C-14 was equally distributed between lipids and protein. Sulfate-S-35 was found primarily (75%) in protein, the rest in metabolic intermediates. Leucine-C-14 was incorporated entirely as the intact molecule

into protein, while over 90% of adenine, guanine, or uracil (16, 17) was incorporated into ribonucleic acids. The incorporation of thymine as such into desoxyribonucleic acid of *E. coli* has been reported (18), although it occurs only to a slight extent (19, 20). Hash (21) found that some free thymine may be catabolized, and label therefrom incorporated into lipid rather than nucleic acid.

If one makes the assumption that incorporation of label from uracil and adenine reflect ribonucleic acid synthesis and C-14 incorporation from labeled leucine indicates protein synthesis, one would conclude from the data obtained that these two general processes are relatively unaffected by a concentration of 2,4-dichlorophenol inhibiting cell division. Sulfate-S-35 (Table V) which also is incorporated into protein is more sensitive to 2,4-dichlorophenol, although this sensitivity may reflect the vulnerability of the sequence of reactions necessary to convert sulfate into its form in amino acids and thus more complex a process than direct incorporation of leucine into protein. Perhaps the incorporation of some label from glucose-C-14 in the presence of the minimal growth inhibiting concentration of 2,4-dichlorophenol represents the nucleic acid and protein synthetic ability still possessed by the cells under these conditions.

Succinic dehydrogenase is located in the cell membrane of *E. coli* and other bacteria (22–25), and the incorporation of succinate into cell substance should involve this enzyme. Much of the evidence relative to the mechanism of action of phenolic germicides points to the cell membrane as a major site of damage, and the finding that incorporation of label from succinate-C-14 into cell material is inhibited by 2,4-dichlorophenol is an expected one in view of the location of this enzyme in the bacterial cell. Similarly, if acetate is converted to lipid and if the latter is synthesized at or near the site in the cell in which it is found, *i.e.*, the peripheral layers of the cell (26), one would expect its metabolism to be inhibited by a germicide acting on the cell membrane. The marked inhibition of incorporation of label from thymine-C-14 is difficult to interpret because of the uncertainty concerning which pathways are followed in its

TABLE IV.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM GLUCOSE-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance	
				0 Time	210 min.
0	0	0.22	72,922	0.46	0.47
0	66.5	0.24	5,236	0.45	0.44
1.25	0	0.53	71,930	0.45	0.79
1.25	66.5	0.29	16,690	0.46	0.57
1.25	133	0.24	1,295	0.46	0.45
5	0	0.63	21,632	0.45	0.79
5	66.5	0.30	4,826	0.46	0.57
5	133	0.22	414	0.46	0.46

[a] A total of 282,420 c.p.m. was added to each culture.

TABLE V.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF SODIUM SULFATE-S-35 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance	
				0 Time	210 min.
0	0	0.18	1,483	0.44	0.45
0	66.5	0.19	748	0.44	0.39
0	133	0.21	552	0.45	0.44
0	266	0.17	541	0.44	0.36
5	0	0.46	57,548	0.44	0.70
5	66.5	0.23	9,693	0.43	0.48
5	133	0.21	673	0.43	0.36
5	266	0.19	450	0.43	0.36

[a] A total of 181,900 c.p.m. was added to each culture.

TABLE VI.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM ADENINE-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance	
				0 Time	210 min.
0	0	0.32	72,964	0.54	0.54
0	66.5	0.30	51,306	0.53	0.49
0	133	0.28	19,998	0.54	0.50
5	0	0.60	138,840	0.53	0.80
5	66.5	0.37	134,887	0.53	0.57
5	133	0.20	39,960	0.53	0.49

[a] A total of 157,070 c.p.m. was added to each culture.

TABLE VII.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM URACIL-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance 0 Time	Absorbance 210 min.
0	0	0.24	96,074	0.48	0.48
0	133	0.24	463	0.47	0.45
0	266	0.21	220	0.48	0.43
0	333	0.23	214	0.47	0.42
5	0	0.53	292,824	0.45	0.78
5	133	0.23	59,450	0.47	0.46
5	266	0.21	6,839	0.47	0.41
5	333	0.22	3,554	0.47	0.41

[a] A total of 422,130 c.p.m. was added to each culture.

TABLE VIII.—EFFECT OF 2,4-DICHLOROPHENOL ON THE INCORPORATION OF RADIOACTIVITY FROM LEUCINE-C-14 BY *E. coli*

Glucose Concn., mg./ml.	2,4-Dichlorophenol Concn., mcg./ml.	Final Cell Concn., mg. dry wt./ml.	Total Radioactivity[a] Incorporated into Cells, c.p.m.	Absorbance 0 Time	Absorbance 210 min.
0	0	0.22	80,670	0.45	0.46
0	133	0.22	2,666	0.44	0.43
0	266	0.23	1,107	0.46	0.42
0	333	0.21	696	0.45	0.41
5	0	0.57	91,210	0.46	0.79
5	133	0.19	48,893	0.44	0.43
5	266	0.23	2,538	0.46	0.41
5	333	0.22	1,012	0.45	0.39

[a] A total of 191,940 c.p.m. was added to each culture.

incorporation into cell material; but if it is catabolized to compounds incorporated into lipid in *E. coli* as it is reported in *B. megaterium* (21), the net result would resemble acetate to the extent that the latter is incorporated into lipid.

One further general effect was the increased uptake of labeled substrate in the presence of glucose, compared to absence of the latter, although growth did not occur in either case due to the 2,4-dichlorophenol. This effect was noted with acetate (Table II) sulfate (Table V), adenine (Table VI), uracil (Table VII), and leucine (Table VIII). Apparently, glucose metabolism must still be possible to some extent at the lowest concentration of 2,4-dichlorophenol inhibiting growth. Perhaps the energy yielded or catabolites produced are protective to certain cell mechanisms.

A difficulty arises in resolving a hypothesis suggesting the cell membrane as the site of action of 2,4-dichlorophenol, with the reports of ribonucleic acid being located in the cell membrane (27) and the relative resistance observed to this phenol compound of C-14 incorporation from labeled uracil and adenine into *E. coli*. Possibly these bases are not incorporated into membrane ribonucleic acid but rather ribosomal ribonucleic acid which would be scattered throughout the cell.

In summary, it was shown that 2,4-dichlorophenol inhibits incorporation of label from a number of substrates to varying degrees, and the pattern of variation seems to be consistent with the hypothesis of cell membrane damage being a primary mechanism of damage by germicidal phenols. Metabolic effects of the latter on isolated metabolic and biosynthetic mechanisms associated with the cell membrane should be investigated to establish a more specific biochemical site of action.

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