# Bacterial Oxidation of Salicylate and Related Antirheumatic Phenolic Acids.* (20533)

BETTY S. ROOF,† THEODORA J. LANNON, AND JOSEPH C. TURNER.

*From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.*

In the course of tests of various antibiotic and bacteriostatic agents for their toxicity to Daphnia(1), it was shown that when the animals were placed in watery solutions of the test materials and were otherwise starved they generally survived at best not more than 2 weeks. A notable exception was observed in experiments with salicylates. Daphnia regularly lived for several weeks, and often multiplied, in M/1000 solutions of salicylate. Inasmuch as this substance acts for the most part as a protoplasmic poison, the mechanism of its beneficial effect in this instance was not immediately apparent but has since been traced to the presence of bacteria which served as food for Daphnia and proved to be capable of growth in media containing as much as M/10 salicylate as the sole carbon source. It was found that entirely similar microorganisms could be recovered readily at any time from dilute solutions of salicylate and ammonia allowed to stand open at the window for a day or two.

Interest in the systematic study of these bacteria stems in large part from the possibility that their biochemical activities might provide information about the metabolic fate of the phenolic acids used clinically as antirheumatic drugs, *viz.*: salicylate, gentisate, and 2:3-dihydroxybenzoate(2). This report concerns an attempt to apply to the problem some of the principles and methods developed in recent years by Stanier(3,4), Happold(5), Evans(6), and others(7-9), for analysis of bacterial fission of other aromatic compounds.

*Materials and methods.* The bacteria used, designated arbitrarily as strains 929A and 616, are Gram-negative aerobic bacilli capable of vigorous growth at 20°C on simple media and in a wide variety of substrates, including citrate. The media contained M/15 phosphate buffer pH 6.5, 0.1% NaCl, 0.1% ammonium sulfate, 0.05% $MgSO_4 \cdot 7H_2O$, and Fe $SO_4$ $10^{-8}$ w/v. After this solution was autoclaved, the aromatic substrates, sterilized by filtration, were added to a final concentration of 0.1%. Strain 929A grew well on salicylate and gentisate and was used for the studies of the oxidation of these compounds. It did not, however, grow on 2:3-dihydroxybenzoic acid.‡ Strain 616 was recovered from a solution of 2:3-dihydroxybenzoic acid allowed to stand open for several days. When isolated in pure culture it was found to grow well only if pantothenate was added in trace amounts. For the cultivation of strain 616, therefore, pantothenate was added to the media in a concentration of $10^{-6}$ w/v.

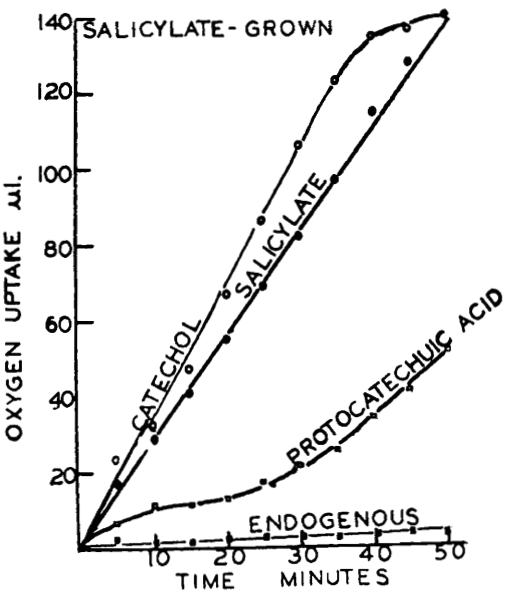


FIG. 1. Oxidation of salicylate, catechol, and protocatechuic acid by strain 929A grown on salicylate.

* This study was supported by a grant from the U. S. Public Health Service (G-3206).

† American Cancer Society Fellow.

‡ The Smith, Kline and French Co. kindly supplied the 2:3-dihydroxybenzoate.

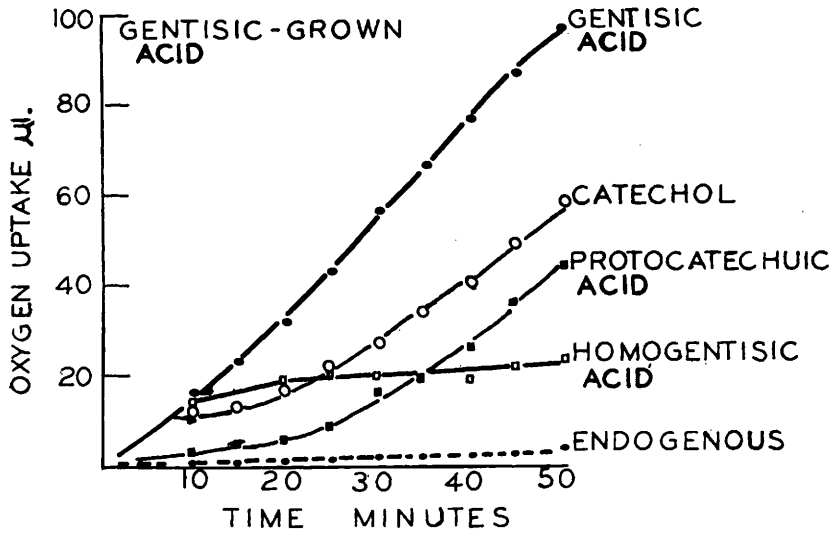


FIG. 2. Oxidation of gentisate, catechol, protocatechuate, and homogentisate by strain 929A grown on gentisate.

The manometric measurements were carried out according to the methods of Stanier(3), using washed bacteria harvested after 24-48 hours' growth on agar plates at 20°C. The bacteria were suspended in M/15 phosphate buffer pH 6.0 to a standard optical density. The Warburg vessels contained in the main compartment 2.0 ml of bacterial suspension, in the side arm 0.2 ml of .01 M substrate. Oxygen uptake was measured in air at room temperature 23-25°C for about 90 minutes.

Qualitative chemical tests were made on filtered aliquots of liquid media. The reaction for $\beta$-diketones was carried out with the reagents of Witter, Snyder, and Stotz(10), while the amounts of gentisate were estimated from the color developed upon addition of Folin's uric acid reagent(11).

*Results.* The pattern of simultaneous adaptation for organisms grown on salicylate is shown in Fig. 1. The rate of oxidation of salicylate is equalled and even a trifle exceeded, by the rate of oxidation of catechol. At the same time, protocatechuate is oxidized very slowly.

It is important at this point to note the essentials of Stanier's(3) postulates: 1) If the dissimilation of A proceeds through a series of intermediates, B, C, D, etc., and if the individual steps in this chain of reactions are under adaptive enzyme control, then growth on A will produce cells simultaneously adapted to A, B, C, D, etc. 2) If growth on A fails to adapt the cells to X then X cannot be a member of the reaction chain. 3) Growth on C adapts to D but not necessarily to A and B.

Applying these ideas here (Fig. 1), it appears that organisms grown on salicylate are simultaneously adapted to catechol, which thus becomes a probable intermediate in the reaction chain. On the other hand there is no adaptation to protocatechuate. Other substrates, not shown in Fig. 1, to which salicylate-trained bacteria demonstrated no adaptation included gentisic acid, homogentisic acid, gallic acid, and pyrogallol. It appears, therefore, that the most probable pathway of salicylate oxidation is in this instance through catechol. Indeed, it has been suggested(4b) that bacterial oxidation of salicylate might involve its transformation to catechol, though an experimental demonstration of this has apparently been lacking.

Bacilli grown on gentisate showed no adaptation to catechol, protocatechuate, or homogentisic acid (Fig. 2). Nor did they oxidize hydroquinone. Entirely similar negative findings were obtained when strain 616 was grown on 2:3-dihydroxybenzoic acid and tested for its adaptation to possible intermediates (Fig. 3). It therefore appeared that

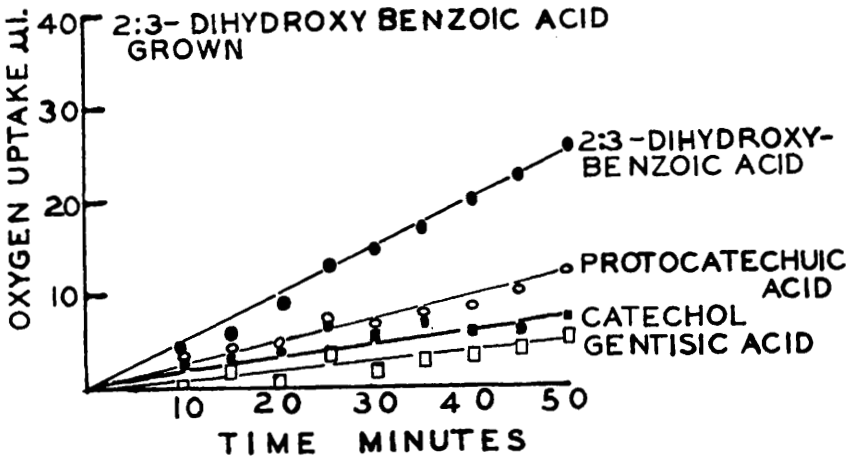


FIG. 3. Oxidation of 2:3-dihydroxybenzoate, protocatechuate, catechol, and gentisate by strain 616 grown on 2:3-dihydroxybenzoate.

the oxidation of gentisate and of 2:3-dihydroxybenzoic acid did not follow any of the pathways yet described for aromatic compounds. It was then hypothesized that the oxidation here might involve simultaneous rupture of the aromatic ring as in the case of homogentisic acid(12-14) with production from gentisate of a straight-chain $\beta$-diketodicarboxylic acid, $C_7H_6O_6$.

Accordingly, qualitative chemical tests were performed to see if any support for this idea could be found. Liquid media containing gentisate as substrate were inoculated with strain 929A and examined at intervals thereafter. It could be shown that as gentisate disappeared there developed some substance or substances forming an orange precipitate on addition of 2:4 dinitrophenylhydrazine in acid solution. Most interesting, however, was the appearance of a reaction with o-phenylenediamine. This was maximal after some 96 hours of bacterial growth, and consisted of 2 distinct visible phases. On addition of filtered medium to an equal part of the o-phenylenediamine reagent(10), a purple precipitate settled out slowly. The supernatant separated from this had the reddish tint which is characteristic of the reaction of $\beta$-diketones(10). Efforts to isolate the substances giving these reactions are incomplete; it has been difficult to separate them from gentisate because of its similar solubility properties. It would clearly be premature to suggest more than that the findings are consistent with the presence of ketones, including $\beta$-diketones, and that these could well be products of the oxidation of gentisate. The possibility was considered that they are not such, but are instead the result of bacterial synthesis. However, media containing other substrates, *e.g.*, salicylate, were examined in the same fashion and showed no comparable reaction with o-phenylenediamine.

Preliminary tests of media containing 2:3-dihydroxybenzoic acid and inoculated with strain 616 give findings entirely similar to those obtained with gentisate, and suggest again the formation of $\beta$-diketones.

*Discussion.* Insofar as the specific problem of the metabolic fate of antirheumatic substances is concerned, the demonstration that salicylate may be oxidized to catechol probably has little or no significance. For, in the first place, it is not known that this reaction can occur in man, although it is said that catechol appears in the urine of horses(15). Secondly, gentisate, an effective antirheumatic agent, appears not to be oxidized to catechol by bacteria; its chemical configuration makes it likely that this would also hold true for mammalian tissues.

On the other hand, it has been known for many years that in man administered salicylate is oxidized in part to gentisate(16). The possibility exists, therefore, that gentisate or some oxidation product of gentisate could be

ultimately responsible for the antirheumatic activity of salicylate. Therefore, the finding by the method of simultaneous adaptation that gentisate is not oxidized through any pathway known for other aromatic substances, *e.g.*, via catechol, protocatechuate, or homogentisate, introduces the necessity of searching out other likely intermediates, including straight-chain keto-acids. Indeed, if the oxidation of gentisate were quite analogous to that of homogentisate one would expect the production of a $\beta$-diketo-, dicarboxylic acid, $C_7H_6O_6$. The qualitative chemical tests carried out so far indicate that such a reaction is entirely possible.

A search through the chemical literature has failed to turn up any helpful information on this score: fumarylpyruvic acid may exist but it has not been synthesized or isolated from natural sources, and must therefore remain for the time being a hypothetical intermediate in the oxidation of gentisate. The problem of the isolation and identification of the oxidation products in the bacterial system must next be undertaken. It may later be possible to apply the findings to a study of gentisate oxidation in man.

*Summary.* 1. The oxidation by bacteria of salicylate, gentisate, and 2:3-dihydroxybenzoate has been examined with the technic of simultaneous adaptation. 2. Catechol appears to be an intermediate in the oxidation of salicylate. 3. Organisms grown on gentisate and 2:3-dihydroxybenzoate show no adaptation to any likely known intermediates. 4. It is suggested that the oxidation of gentisate may be analogous to that of homogentisate, with formation of a straight-chain $\beta$-diketo-dicarboxylic acid.

The authors also wish to thank Drs. K. Meyer and C. Ragan for many helpful suggestions and some of the chemicals, and Dr. J. MacLennon for advice in bacteriology.

1. Turner, J. C., and Lannon, T. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 684.

2. Michotte, L., and Ranaux, R., *Acta Physiother. et Rheumatol. Belg.*, 1952, v7, 142.

3. Stanier, R. Y., *J. Bact.*, 1947, v54, 339.

4. a. ———, *J. Bact.*, 1948, v55, 477; b. cites Tausson, W. O., *Planta* 4, 5, and 7, 1927-29.

5. Happold, F. C., *Biochem. Soc. Symposia*, 1950, No. 5, 85.

6. Evans, W. C., Smith, B. S. W., Linstead, R. P., and Elridge, J. A., *Nature*, 1951, v168, 772.

7. Sleeper, B. P., and Stanier, R. Y., *J. Bact.*, 1950, v59, 117.

8. Kilby, B. A., *Bioch. J.*, 1948, v43, V.

9. Suda, M., Haygaishi, O., and Oda, Y., Osaka, Japan, *Univ. Med. School J.*, 1950-51, v2, 21.

10. Witter, R. F., Snyder, J., and Stotz, E., *J. Biol. Chem.*, 1948, v176, 493.

11. Smith, M. J. H., *J. Pharm. and Pharmacol.*, 1950, v2, 439.

12. Suda, M., and Takeda, Y., Osaka, Japan, *Univ. Med. School J.*, 1950-51, v2, 37.

13. ———, *ibid.*, p. 41.

14. Ravdin, R. G., and Crandall, D. I., *J. Biol. Chem.*, 1951, v189, 137.

15. Fieser and Fieser, *Organic Chemistry*, 1944, D. C. Heath & Co., Boston, Mass., 638.

16. Angelico, F., *Arch. farmacol. sper.*, 1921, v31, 8; cited by Kapp, E. M., and Coburn, A. F., in *J. Biol. Chem.*, 1942, v2, 549.

Received July 28, 1953. P.S.E.B.M., 1953, v84.

## Glucosamine and Leukemia.* (20534)

ARTHUR A. MARLOW AND GRANT R. BARTLETT.

*From The Scripps Metabolic Clinic, La Jolla, Calif.*

Recently Quastel and Cantero(1) reported an inhibition of mouse sarcoma 37 by glucosamine. The rationale of their study was as follows. Hexokinases are able to phosphorylate glucosamine *in vitro*(2,3), the reaction being competitive with glucose. Tumors, presumably being particularly dependent on glycolytic energy sources, might be selectively influenced by a glucosamine block of glucose

* Supported in part by the P. C. Pinson Fund.