Summary. 1. Prostatic tissue obtained surgically from 100 consecutive males, who had lived for many years in an endemic area of brucellosis in Minnesota, were cultured for brucella and in no instance were brucella isolated. 2. On the basis of historical evidence, as well as positive intradermal tests and the presence of brucella agglutinins, many of the individuals had been exposed to brucellosis.

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Reversal of Aminopterin Inhibition in the Chick Embryo with the *Leuconostoc citrovorum* Factor.* (18094)

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In an earlier study(1), minute amounts of aminopterin were shown to inhibit development of the chick embryo. Under the conditions tested, this inhibition could be partially alleviated by thymidine, and more effectively by thymidine plus hypoxanthine desoxyriboside but not by folic acid, vitamin B_{12} or several other substances. The conclusion was drawn that aminopterin inhibited synthesis of thymidine and one or more of the purine desoxyribosides by the embryo, and that by eliminating the need for these synthetic reactions during the early stages of embryonic development, added desoxyribosides counteracted the inhibitory action of aminopterin. Since aminopterin appears to act as an anti-folic acid, the direct or indirect participation of folic acid in synthesis of these desoxyribosides was indicated. Recent evidence(2-6) indicates that the *Leuconostoc citrovorum* factor (CF) is a metabolically active form of folic acid, and that it can be formed synthetically from folic acid(5,6). Concentrates of this substance counteract aminopterin inhibition of *Leuconostoc citrovorum*(4). Amounts of CF previously available for testing failed to alleviate aminopterin inhibition of the chick embryo; it was pointed out, however, that larger amounts might prove effective(1). It is shown below that adequate amounts of CF partially counteract aminopterin inhibition of the chick embryo under conditions where folic acid is ineffective.

Experimental. Procedures used in injecting solutions and incubating eggs were exactly similar to those described previously(1,7). All injections made prior to incubation totaled 0.1 ml. Those made at 3 days totaled 0.2 ml.

Results. Table I presents the results obtained when aminopterin was injected alone or together with CF concentrate, folinic acid (6), or folic acid just prior to incubation. A pronounced effect of CF in reversing the inhibitory effect of aminopterin is evidenced not only by the increased hatch obtained in lots receiving adequate amounts of this substance, but by the greatly decreased number of embryos that died early in the incubation period. Larger amounts of the concentrate are required for this purpose in those lots receiving 20 μg than in those receiving 10

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1. Snell, E. E., and Cravens, W. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 87.

2. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1949, v181, 871.

3. Bond, T. J., Bardos, T. J., Sibley, M., and Shive, W., *J. Am. Chem. Soc.*, 1949, v71, 3852.

4. Sauberlich, H. E., *Arch. Biochem.*, 1949, v24, 224.

5. Nichol, C. A., and Welch, A. D., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 52.

6. Shive, W., Bardos, T. J., Bond, T. J., and Rogers, L. L., *J. Am. Chem. Soc.*, 1950, v72, 2817.

7. Cravens, W. W., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 73.

8. Bardos, T. J., Bond, T. J., Humphreys, J., and Shive, W., *J. Am. Chem. Soc.*, 1949, v71, 3852.

TABLE I. Counteraction of Aminopterin Inhibition of Chick Embryo by CF. Injections at zero days incubation.

Substance inj., amt	No. of eggs	% embryos dying at 0-5 days	% embryos dying at 6-20 days	% hatch
None	19			100
Water (0.1 ml)	20	5.0	15	80
Aminopterin, 20 μg	19	89.5	10.5	
" 20 μg				
+ CF,* 300,000 units	10	100		
+ CF, 600,000 "	20	85		15
+ CF, 1,200,000 "	20	65	10	25
+ CF, 3,000,000 "	20	35	30	35
Aminopterin, 10 μg	10	90		10
" 10 μg				
+ CF, 600,000 units	10	90		10
+ CF, 1,200,000 "	10	50		50
+ CF, 3,000,000 "	10	20	20	60
+ Folinic acid,† 10 μg	8	62.5		37.5
+ " " 500 μg	10	100		

* The CF concentrate used was kindly supplied by Drs. T. H. Jukes and H. P. Broquist of Lederle Laboratories. It contained 30,000,000 CF units per ml; and 300,000 units per mg of solids. One CF unit is the amount required per ml of medium to permit half maximum growth of *Leuconostoc citrovorum* under defined conditions(2).

† The sample of folinic acid, kindly supplied by Dr. W. Shive, was synthesized from folic acid(6) and subsequently fractionated until free of folic acid and of formylfolic acid (personal communication). This product promotes growth of *Leuconostoc citrovorum* under the same conditions as CF, and it appears likely that the two products are identical(6,8). The absolute purity of the product tested is not known.

μg of aminopterin. In confirmation of previous results(1) folic acid was ineffective in alleviating the inhibition when injected at zero days.

Assay with *Lactobacillus delbrueckii* 730, an organism that requires thymidine for growth(9,10), showed the CF concentrate to be free of this desoxyriboside. Its effects thus cannot be ascribed to this substance or to folic acid. The limited amount of folinic acid available showed definite activity in counteracting aminopterin inhibition, and since this substance was prepared synthetically from folic acid and has high CF activity, the evidence is conclusive that the favorable effects of the CF concentrate are actually due to the CF that it contains.

The effects on hatchability of similar injections made after 3 days of incubation are shown in Table II. The effect of CF in overcoming the toxic effects of aminopterin is unmistakable. Folic acid and formylfolic acid appear to be slightly effective under these conditions, but much less so than CF.

TABLE II. Effect of Aminopterin and Related Compounds on Development of 3-Day Embryos.

Substance inj., amt	No. of eggs inj.	% hatch
None	10	100
Water, 0.2 ml	18	94.5
Aminopterin, 20 μg	19	21
" 20 μg		
+ CF, 600,000 units	10	30
+ CF, 1,200,000 "	10	50
+ CF, 3,000,000 "	17	76.4
+ CF, 6,000,000 "	10	70
+ Folic acid, 500 μg	18	33.3
+ Formylfolic acid, 500 μg	10	40

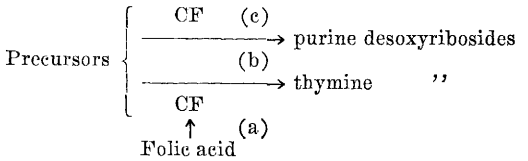
Discussion. It was observed previously that mixtures of thymidine and hypoxanthine desoxyriboside partially counteracted the inhibitory effects of aminopterin on growth of the chick embryo under conditions where folic acid was ineffective. The present data demonstrate that similar reversals are effected by the *Leuconostoc citrovorum* factor (CF), a substance which is derived from folic acid in intact animals(11), in liver slices(5), and by non-enzymatic, chemical

9. McNutt, W. S., and Snell, E. E., *J. Biol. Chem.*, 1950, v182, 557.

10. Kitay, E., McNutt, W. S., and Snell, E. E., *J. Bact.*, 1950, v59, 727.

11. Sauberlich, H. E., *J. Biol. Chem.*, 1949, v181, 467.

means *in vitro*(6). These data are most readily explained by assuming (a) that conversion of folic acid to CF is a necessary preliminary step to its participation in catalysis of certain synthetic reactions, (b) that among the synthetic reactions for which CF is essential are syntheses of thymidine and one or more of the purine desoxyribosides, and (c) that the latter reactions are the first to be inhibited by aminopterin in the developing embryo. These postulates are expressed diagrammatically as follows:



The arrows may represent more than a single chemical transformation.

The growth-promoting action of CF (or folinic acid) for organisms which require folic acid(8) is thus readily explained, since these organisms must normally convert folic acid to CF before it can be utilized. Existence of organisms such as *L. citrovorum,* which cannot utilize folic acid readily in place of CF is predicted by this scheme; such organisms fail to carry out conversion (a) readily. The scheme also explains the enhanced activity of CF, as compared with folic acid, in counteracting growth inhibition by methylfolic acid(3) and by aminopterin (cf. 4, 12, and this paper); for precursors of a metabolite are frequently much less effective than the metabolite itself in counteracting the inhibitory effects of an antimetabolite(13,14). Finally, the scheme adequately accounts for reversal of aminopterin inhibition by thymidine or by thymidine and hypoxanthine desoxyriboside, observed both in bacteria(4,15) and the chick embryo(1); for aminopterin, at the concentrations used, is visualized as blocking primarily reactions (b) or (b) and (c). When the desoxyribosides are supplied preformed, the blocked reactions are no longer required for growth.

The relative activity of CF in reversing the toxic effects of aminopterin for various organisms varies widely. For *L. citrovorum,* 11 units of CF counteracted the toxic effects of 1-4 μg of aminopterin(4); in mice, about 20,000 units of CF was required for this purpose† (12), while the corresponding figure for the chick embryo is 100,000-200,000 units or more. The best estimates available indicate that 1 unit of CF is equivalent to 0.1-0.2 mμg of folic acid in growth-promoting activity for *Streptococcus faecalis.*‡ Assuming the two products to have equimolar activities and approximately equal molecular weights, 200,000 units of CF would be equivalent to about 20 μg of the pure vitamin.

Summary. Concentrates of the *Leuconostoc citrovorum* factor (CF) and of "folinic acid" partially counteract the inhibitory action of aminopterin for the chick embryo. Folic acid and formylfolic acid were ineffective or only slightly effective under the same conditions. The magnitude of the effects obtained with CF were similar to those obtained previously with mixtures of thymidine and hypoxanthine desoxyriboside(1). The viewpoint is developed that conversion of folic acid to CF is a necessary preliminary to the catalytic action of the former compound, that synthesis of thymidine and one or more of the purine desoxyribosides are among the synthetic reactions for which CF (or folic acid) is required, and that these synthetic reactions are those inhibited in the chick embryo by small amounts of aminopterin. The great variation that exists in the ability of CF to counteract aminopterin inhibition in various organisms is pointed out.

12. Broquist, H. P., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.,* 1950, in press.

13. Volcani, B. E., and Snell, E. E., *J. Biol. Chem*, 1948, v174, 893.

14. Shive, W., and Macow, J., *J. Biol. Chem.,* 1946, v162, 451.

15. Franklin, A. L., Stokstad, E. L. R., Hoffman, C. E., Belt, M., and Jukes, T. H., *J. Am. Chem. Soc.,* 1949, v71, 3549.

† Broquist *et al.*(12) reported that 100,000 units of CF counteracted the effect of 10 μg of aminopterin in mice. Their unit is approximately twice that of Sauberlich and Baumann(2), employed in this paper.

‡ Private communication from Dr. H. P. Broquist.

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