the bone analyses in showing an effect at the level of 8 ppm. The variable data reveal no differences between the 2 sources of fluorine, nor for the different lengths of time on experiment. The teeth of the animals from the groups killed at 56 days were combined and analyzed for fluorine with the following results:

	Fluorine, ppm.
Controls	81
Sodium fluoride, 8 ppm	228
Bone meal, 8 ppm	220
Sodium fluoride, 12 ppm	246
Bone meal, 12 ppm	276

These data confirm the results of the bone analyses in showing the distinct effect of the small fluorine additions, in revealing no differences between the two supplements, and in showing a greater effect at the higher level.

It cannot be stated, without further study, whether the pigment changes and increases in fluorine content obtained in these experiments represent a definite structural injury. The diet fed our stock rats has regularly contained one per cent of bone meal. In a recent examination of our entire colony of mature animals most of them showed a slight lightening of the enamel, the others showing no detectable change. Analyses of the bones of animals approximately 500 days of age gave an average fluorine value of 335 ppm.

Summary. The data here reported show clearly that fluorine additions of 8 to 14 ppm. to a basal diet containing 3 ppm. results in growing rats in increases in the fluorine content of the bones and teeth, which are marked and roughly proportional to the level fed. They show sodium fluoride and bone meal to be equally effective in causing these changes. They suggest that analysis for fluorine is a more sensitive measure than the enamel pigment changes which are also noted at these low levels. The data indicate that the fluorine content of the bones and teeth increases with age during growth.

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Purification of Tetanic Toxin.

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The methods used in the purification of tetanic toxin were based on the general principles outlined in a previous paper on the purifi-

cation of diphtherial toxin.[1] The crude toxins were prepared in meat-infusion containing 2% of Bacto-peptone, and 1% dextrose. Cultures of *Cl. tetani* were grown 14 days under vaseline and filtered. Lots 4 and 5 were about one year old, and lots 6 and 8 were fresh when the purification was started.

Purification. The toxic preparations should be kept in a refrigerator except when reagents are added or when being centrifuged. The optimal proportions of reagents vary somewhat with different lots of toxin. These variations have been indicated in the directions for the process given below. Amounts are for the purification of one liter of crude toxin.

1. To one liter of crude toxin 100 to 125 cc. of 10% solution of ferric ammonium sulphate are added gradually. The pH is kept between 5.5 and 6.0 by the addition of normal sodium hydroxide. The resulting precipitate will dissolve in an excess of the ferric salt below pH 5.5. After settling for a few hours the precipitate is collected and washed with distilled water. The toxin is then eluted with 400 cc. of 2.5% sodium citrate at a pH of 7.5 to 8.0 for 3 days or until most of the precipitate has dissolved. The insoluble residue is centrifuged down and the pH of the supernatant adjusted to 7.0.

2. Four hundred cc. of the eluted toxin are diluted to one liter and the calculated concentration of sodium citrate adjusted to 1.5 to 2.5%. Three hundred to 450 cc. of 5% cadmium chloride are added until no further precipitation occurs and the pH is near 5.8. After 2 hours the precipitate is centrifuged, washed, and emulsified in about 300 cc. of 4% sodium chloride. Most of the toxin is dissolved out of the precipitate if the pH of the suspension is adjusted to 6.8 with a few drops of normal hydrochloric acid, but very little toxin dissolves above pH 7.5. After standing over night at a pH of 6.8 the insoluble residue is centrifuged down. The clear yellow supernatant contains the toxin.

3. To 300 cc. of this supernatant are added 15 cc. of 20% sodium citrate and then 2.5% solution of neutral lead acetate until the solution is quite opalescent. (About 50 cc. of lead acetate.) The precipitate gradually increases in amount and after settling over night is centrifuged down and discarded. If not too much lead acetate has been added the toxin will remain in the supernatant. This step is included here because it is advantageous in removing inactive protein and colored substances from certain preparations, but it may be omitted.

[1] Eaton, M. D., *J. Bact.*, 1936, **31**, 347, 367.

4. On the solutions of toxin resulting from steps 2 or 3 precipitation with cadmium chloride is repeated by adding an equal volume of 5% cadmium chloride and adjusting the pH to 7.8 with 5% barium hydroxide. After an hour the precipitate is centrifuged down and eluted with 2% sodium bicarbonate adjusted to a pH of 7.8 by the addition of phosphoric acid. After standing over night the residue is centrifuged down. The bicarbonate solutions of toxin must be kept in tightly stoppered containers to prevent loss of carbon dioxide. If the solution becomes strongly alkaline the toxin will be destroyed. The clear almost colorless elution of the cadmium precipitate contains the purified toxin.

Some of the preparations were dialyzed in cellophane against running water for 48 hours to remove salts and residual peptones. A partial precipitation of the toxin may occur during dialysis. Sodium bicarbonate solutions of toxin should be buffered at pH 7.0 with acid sodium phosphate before dialysis.

Results of Purification. At least 50% of the total M.L.D. of tetanic toxin should be recovered after each of the 4 steps of the purificatory process just described. The resultant yields of purified toxin are 10 to 30%.

Representative results of the procedure are presented in Table I. The M.L.D. was determined by subcutaneous injection at the groin. Ascending tetanus resulted from all active preparations. Since some of the purified toxins seem to be partially inactivated by dilution, 0.1 cc. of the appropriate dilution was injected in preference to 1.0 cc. of a 10-fold greater dilution. Like crude tetanic

TABLE I.
Purification of Tetanic Toxin Expressed in Terms of Nitrogen per M.L.D.

Crude toxin No.	Purificatory process*	M.L.D. per cc. for mice	M.L.D. per cc. for guinea pigs	Mg. N† per cc.	Mg. x 10–6 N/M.L.D. mice	Mg. x 10–6 N/M.L.D. guinea pigs
4	crude	500	—	2.16	4,300	—
4	1, 2	2,500	—	0.305	122	—
5	crude	3,000	1,500	2.90	960	1,930
5	1, 2	10,000	—	0.260	26	—
5	1, 2, 3, 4	8,000	4,000	0.053	6.6	13
5	1, 2, 3, 4, D	2,000	1,500	0.027	13.0	18
6	crude	5,000	2,000	2.32	460	1,160
6	1, 2	2,500	1,000	0.044	17.0	44
6	1, 2, 4	10,000	5,000	0.046	4.6	9
8	crude	4,000	—	2.65	650	—
8	1, 2, D	2,000	—	0.022	11.0	—
8	1, 2, 3, 4	—	1,500	0.018	—	12

* The steps of the procedure used in purification are designated by numbers corresponding to those in the text. D = dialysis.

† Nitrogen determined by Pregl micro-Kjeldahl method. In the last 2 columns nitrogen per M.L.D. is expressed in millionths of a milligram.

toxin, the purified toxins are 5 to 10 times as toxic for guinea pigs, per gram of body weight, as for mice.

The process separates tetanic toxin from 99.0 to 99.5% of the nitrogenous impurities. The dried organic residue from the purified dialyzed toxin contains about 12% of nitrogen. Twenty-five to 50% of the total nitrogen in purified toxin is precipitated by 5% trichloracetic acid as protein. At least part of the remaining nitrogen is in proteoses or peptones. The purified preparations when sufficiently concentrated give positive biuret, Millon, xanthoproteic, diazo, and sulphur reactions. In some the Molisch test is positive, in others, doubtful.

For a 500 gm. guinea pig the M.L.D. of the purest preparations contains 9 to 18 millionths of a milligram of nitrogen. This represents a fatal dose of 0.00015 to 0.00030 mg. of dried organic material per kilo of guinea pig. The lethal dose of purified diphtherial toxin previously reported[1] was 0.00040 to 0.00060 mg. per kilo.

The preparation of highly active dried tetanic toxin by fractionation with ammonium sulphate and dialysis has been reported by London and Aristovsky[2] and by Brieger and Cohn.[3, 4] The methods used by these authors have not been as successful in our hands as those described in the present paper.

Although the purified preparations are obviously still a mixture of substances, it seems likely that the purification of more powerful crude toxins than those used in the present work by improved methods would produce even more active purified toxins.

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[2] London, E. S., and Aristovsky, V. M., *Compt. rend. Soc. de Biol.*, 1917, **80**, 756.

[3] Brieger, L., and Cohn, G., *Z. f. Hyg. u. Inf.*, 1893, **15**, 1.

[4] For a review of earlier purification experiments see: Kolle, W., Kraus, R., and Uhlenhuth, P., *Handbuch der Pathogenen Mikroorganismen*, Dritte Auflage, 1929, **2**, 391, 531; 1928, **4**, 1035.