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The program of the Fifth Annual Conference of the I. S. F. R. in Oxford, England, April 8 through April 10 will feature two symposiums, namely: Fluorosis in Domestic Animals on April 9th and Organofluorides on April 10th. Other subjects on the program will deal with metabolic studies in endemic fluorosis, histopathology of skeletal fluorosis, physical properties of bones in skeletal fluorosis, fluoride damage in plants, biological effect of fluoride compounds, and fluoride's effect on kidneys. For accomodations kindly contact Prof. H. M. Sinclair, International Institute of Human Nutrition, High Street, Sutton Courtenay, Berks. 0X14 4AW, England.

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FLUORIDE is listed in
Current Contents Agricultural
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EDITORIAL

IS FLUORIDE AN ESSENTIAL ELEMENT?

In 1933 Sharpless and McCollum (1) attempted to determine by means of experiments on rats whether or not fluoride is essential for nutrition. After the rats had received a semi-purified diet containing casein, starch, butterfat, yeast and salts for 120 days the rats' femurs contained about 150 μg of fluoride. In other words, the investigators were unable to devise a diet low enough so that the animals receiving the diet were suitable as nonfluoride controls.

In 1934, Phillips, Hart, and Bohstedt (2) carried out similar experiments with a mineralized milk diet which contained only 0.1 to 0.2 ppm fluoride. Nevertheless, at 140 days of age, they found several hundred micrograms fluoride in the carcasses of the control animals. In subsequent experiments Evans and Phillips (3), using the same type of milk diet, were unable to establish the essentiality of fluoride in nutrition. In 1951 and 1954 Muhler (4, 5) showed that rats fed diets with not more than 0.1 ppm of fluoride had stored in their femurs between 23 and 39 ppm of fluoride by the time they were 80 days of age. Muhler demonstrated therefore that no diet was low enough in fluoride to permit conclusions concerning the essentiality of fluoride.

In 1953 McClendon and Gershon-Cohen (6) attempted to solve the question by providing rats with a diet which consisted mainly of corn and sunflower seed. The plants from which the grain was obtained were grown in rainwater reported to contain only 0.0002 to 0.0004 ppm of fluoride. The control animals received a diet compounded of ordinary soil-grown crops. No data were given on the fluoride content of either the diet or the animals. Because the "deficient" animals were smaller in size and had a higher incidence of dental caries, the authors felt that fluoride is an essential element.

In new attempts to settle this question Maurer and Day in 1957 (7) used a commercial diet consisting mainly of casein, DL-methionine, corn oil, vitamin mixture, salts and corn starch. They eliminated fluoride from this diet by various procedures which they described in detail. The diet was estimated to contain no more than 0.007 ppm fluoride. Their studies embraced 110 rats representing four generations which were conceived, born, and weaned on the highly purified diet which was almost totally lacking in fluoride. All animals were weaned at 25 days of age. Controls received water containing 2 ppm fluoride as sodium fluoride. Most animals were observed for a period of 150 days, but two pairs of animals, one on the purified and the other on the "supplemented" diet were observed for 325 days. After 160 days the fluoride content of the femurs was often too small to be measured. Even the rats which had been on the diet for 325 days had only approximately 2 ppm fluoride in the femurs. Maurer and Day found no impairment in reproduction in rats on the fluoride-purified diet nor could the rats be distinguished from those receiving a 2 ppm fluoride supplement in drinking water either with regard to their growth or gross appearance. The teeth and the levels of alkaline and acid phosphatase ac-

tivity showed no significant differences in the two groups. Maurer and Day concluded that under these experimental conditions fluoride may not have any value in nutrition of rats or even in the maintenance of dental health.

Recently, Messer, Armstrong, and Singer (8) carried out experiments on two groups of mice, 55 in each. The "fluoride supplemented" females received 50 ppm of fluoride in deionized water. Litter production was observed over a 25 week period to a maximum of 4 litters. Composition of the basic diet other than fluoride and its preparation were not described, but its fluoride level ranged between 0.1 to 0.2 ppm, a level at which Muhler had reported storage of 23 to 39 ppm of fluoride in the femurs.

The female animals on the low fluoride regime showed a progressive decline in litter production; those with fluoride supplements reproduced normally and at consistent intervals. When females whose fertility had been impaired received a fluoride supplement their reproductive capacity was restored. The authors therefore considered fluoride essential for fertility in mice.

A different kind of experiment involving a single massive dose was recently recorded by Devoto et al. (9). They injected intraperitoneally into five groups of 6 rats each 1, 5, 10, 15, or 20 mg NaF diluted in saline solution per kg body weight from the 10th to 18th day of pregnancy. Another five groups of six rats each received injections of NaF subcutaneously into the back following the same experimental design. A control group comprised of twenty rats was injected with saline solution. No attention was given to the fluoride content of the diet nor to its accumulation in the rats' carcasses. The fetuses of all fluoride-treated animals died in the uterus. The placentae of these animals were found to be necrotic.

Devoto's team administered repeated well defined doses of fluoride. In the experiments by Maurer et al. and in those by Messer et al., the addition of 2 ppm and 50 ppm sodium fluoride respectively to drinking water extended over four generations. The amount of fluoride consumed by the rats was dependent upon the amount of water the animals drank. This amount is subject to wide variations: polydipsia is a common manifestation associated with fluoride intake. The oral route of administration would not be likely to lead to changes as drastic as those encountered by Devoto et al. in their experiments.

On the other hand, the studies of Messer et al. with mice resemble closely those by Maurer and Day with rats. No changes in fertility occurred among the Maurer and Day animals which received the diet highly purified of fluoride whereas Messer et al. reported fertility impairment from the diet containing 0.1 to 0.2 ppm fluoride. In fact Maurer et al. stated that "all mated females became pregnant and they gave birth to live pups." Such a discrepancy between the findings in the two series of experiments requires an explanation.

Devoto cautioned against extrapolating to man results obtained in rats.

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ENDEMIC FLUOROSIS IN PUNJAB

I. SKELETAL ASPECT

by

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Patiala, India

SUMMARY: In extensive surveys of ten villages from a known hyperendemic fluorosis area of Punjab namely the Bhatinda and Sangrur Districts an effort was made to examine each individual and to take X-rays on as many as possible. A very high incidence of dental (44 to 81 %) and skeletal (2.8 to 81 %) fluorosis was observed but no strict correlation between the two manifestations could be established. Crippling and neurological fluorosis occurred much more frequently in men than in women.

The chief factors which determine fluoride toxicity are the fluoride concentration in drinking water and the duration of exposure to this hazard. Yet in spite of identical concentrations of fluoride in water, variations in the incidence of the disease occurred. They were related to other chemical constituents of drinking water which are protective against the development of fluorosis such as magnesium, calcium and total hardness. Physical stress which was chiefly responsible for the severity of disease in men was also responsible for the neurological complication. Malnutrition played no etiological role in this disease. Food grown in the fluorotic soil did not provide a significant quantity of fluoride.

Methods and Material

The study has been conducted in 10 villages of districts Bhatinda and Sangrur, two of the hyperendemic districts of Punjab. All these villages are located within a 45 miles (72.42 km) radius of Bhatinda city. The climate of this area is hot and dry. In summer, temperature remains above 100° F (37.6° C) and often exceeds 110° F (43.3° C). Rainfall is rather scanty. The majority of the population surveyed is agricultural by occupation and is given to hard manual labor all year round.

The survey covered a total population of about 9000, 6212 of which belonging to all age groups were examined clinically through a house to house survey. A specially-designed form was used to record data regarding age, sex,

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length of residence in the village, source of drinking water supply, complaints and findings on clinical examination. This was followed by representative radiological examination of adults over 21 years of age to evaluate the prevalence of skeletal fluorosis in individual villages. X-rays of both arms were done with the aid of 70mm mass miniature mobile radiography unit. Interosseous membrane calcification of the forearm bones was considered definite evidence of skeletal fluorosis. The X-ray films were graded into positive, indeterminate (borderline) and negative.

Nutritional assessment was made through clinical examination and was supported by representative dietary surveys by the questionaire method. The data so collected was analyzed to work out the daily intake of different constituents according to I. C. M. R. special series No. 48.

Drinking water sources in these villages are mostly hand pumps; underground water has a depth of about 20 to 40 feet. All these drinking water sources were analyzed for their fluoride content and such other inorganic chemical constituents as calcium, magnesium, chlorides, sulphates, phosphates, alkalinity, hardness and total solids. Similarly foodstuffs grown in the endemic villages and samples of endemic soil were also analyzed for fluoride. Furthermore in three villages 24 hour urine samples of 68 subjects were assayed for their fluoride content.

All fluoride estimations were made according to I. C. M. R. special series No. 57, whereas other inorganic constituents of water were determined according to the 1960 edition of standard methods for examination of water and waste water.

Results

Though analysis of all the surveyed population is available the data pertaining to children between the ages of 5 to 15 and of adults over 21 years-old were found to be most informative. They are herewith presented. The terms "Children" and "Adult" henceforth apply to these specific age groups unless otherwise specified.

It is seen that whereas 91.6 to 97.4% of adult males had been local residents, only 2.9 to 8.7% adult females had resided in these villages since birth. The rest had moved there after marriage. Because adult males were subjected to the least variations of extrenuous influence, this group was considered representative of the village concerned whenever comparisons were made in this study.

Observations

A striking feature in the current study was a rise in incidence of skeletal fluorosis in the villages in direct relation to the rise in the fluoride level of drinking water associated with a proportionately greater number of edentulous subjects as well as a higher incidence of premature loss of teeth. This feature was clearly manifest in adult males but less frequently in females.

Skeletal manifestations were observed in adult subjects exclusively; children did not show any skeletal involvement. Table 1 shows the relative frequency of these manifestations in the current surveys which were in the form of limitation or movement. Clinically the subjects experienced restriction of movements in the spine mainly in the cervical region but also in the lower back, shoulder joint, hip and knee. Such deformities as kyphosis and exostosis were evident. The incidence of these features is shown in Table 1.

TABLE 1

Skeletal Manifestation of Fluorosis in Adults (Above the Age of 27 Years)

		Male					Female				
Village	in water oride ppm	Mber fluined	Numptoms exm%	symitation of al move- : %	Lim spin ment	Exo rmities %	Def ined	Num ptoms exm%	symitation of al move- t %	Lim spinstoses % mer	rmities %	Def
Mandi Baretta	0.73	120	30.0	32.5	-	0.8	169	36.7	29.6	-	-	
Kooriwara	2.25	48	29.2	33.3	-	-	30	33.3	33.3	-	-	
Gurney Kalan	2.45	142	35.9	45.8	15.4	7.7	208	31.3	29.8	5.7	7.7	
Ganza Dhanaula	4.3	77	31.2	36.4	3.9	8.9	75	36.0	18.7	-	3.8	
Bajakhana	5.02	145	64.9	53.1	18.9	9.6	216	52.3	35.2	2.3	7.8	
Rajia	5.2	117	60.7	52.9	35.0	13.6	206	29.0	23.0	7.0	2.5	
Village Baretta	5.49	132	54.5	43.4	6.8	4.5	173	44.9	30.5	0.6	3.5	
Rorki	7.02	90	58.9	48.9	37.0	15.5	147	42.9	30.6	8.2	8.2	
Saideke	8.26	50	46.0	28.0	26.0	14.0	74	33.8	13.5	9.4	1.3	
Khara	9.4	190	72.1	52.1	29.4	18.4	173	63.4	30.0	2.3	2.1	

The exostotic bony nodules could be palpated clinically at a number of superficial bony sites usually at the attachment of muscles or fascia namely near the tibial tubercle, the dorsum of foot, the superficial ulnar border, the medial border of the scapula and soleal line along the medial border of the tibia. The latter was the most common site and constituted a very useful diagnostic sign. The importance of this sign was realized during the course of the investigations which accounts for the variability of the figures in Table 1. Exostoses were more prevalent in males than in females and in those doing heavy manual work.

Radiological survey: A total of 1848 persons above 21 years of age were studied radiologically. The results of the survey are given in Table 2.

Although in general there is a trend of a parallel incidence of skeletal fluorosis with increasing fluoride in drinking water, there are many exceptions which are shown in Table 3.

Females were observed to be less affected in all phases of life (Table 4). The incidence of skeletal fluorosis in females is also significantly lower in most of the villages (Khara, Rorki, Bajakhana, Ganza Dhanaula, Gurney Kalan and Koori-wara) whereas in 3 villages the incidence is almost equal (Table 2).

Table 4 illustrates that skeletal fluorosis increases with advancing age both in males and in females reflecting the influence of the duration of fluoride exposure on the development of skeletal fluorosis.

Also in the hyperendemic villages a higher percentage of skeletal fluorosis was seen in the younger ages, whereas no cases of skeletal fluorosis were present in the 21-30 year-old group in the low endemic village (Table 5).

Factors Influencing Toxicity

The current study was undertaken to determine the known and unknown factors which influence the development of skeletal fluorosis. The following points have been brought to light:

1. Total Fluoride Intake: Fluoride content of drinking water is the single primary factor which determines the severity of manifestations of fluorosis. Certain modifying factors however do exist (Table 2 and 3).

a. Amount of water consumed: In the endemic area of Punjab the climate is hot and dry. A farmer working in the fields doing hard manual labor during the summer months drinks a large quantity of water. Therefore correspondingly increasing quantities of fluoride are also consumed.

b. Fluoride Ingestion Through Food: Forty-three samples of food were analyzed (cereals, pulses, common salt) for their fluoride content. It was not determined that these food items would contribute a significant amount of fluoride compared to that ingested through drinking water alone.

2. Duration of Exposure: That duration of exposure is a well known factor influencing the incidence of fluorosis has been shown in the current study. Table 4 and 5 demonstrate that the longer the duration of exposure, the more pronounced is the toxic effect of fluoride.

3. Nutritional factors: Although given much importance by earlier workers, nutritional facts do not seem to influence the development of skeletal fluorosis. The subjects of the current study were well developed and well nour-

TABLE 2

Radiological Study of 10 Villages (of adults over the age 21)

Village	Water fluoride ppm	Total cases X-rayed	MALE				FEMALE			
			Number of cases evaluated	Positive percentage	Undetermined percentage	Negative percentage	Number of cases evaluated	Positive percentage	Undetermined percentage	Negative percentage
Mandi Baretta	0.73	165	70	2.8 (2)*	8.6 (6)	88.6 (62)	95	4.2 (4)	12.6 (12)	83.2 (79)
Kooriwara	2.25	48	30	40.0 (12)	6.7 (2)	53.3 (16)	18	22.2 (4)	22.2 (4)	55.6 (10)
Gurnay Kalan	2.44	112	67	19.6 (13)	28.4 (19)	52.0 (35)	45	11.1 (5)	8.9 (4)	80.0 (36)
Ganza Dhanaula	4.2	63	38	26.3 (10)	39.5 (15)	34.2 (13)	25	16.0 (4)	36.0 (9)	48.0 (12)
Bajakhana	5.09	146	98	46.9 (46)	28.6 (28)	24.5 (24)	48	39.6 (19)	29.2 (14)	31.2 (15)
Rajia	5.2	285	180	52.2 (94)	28.9 (16)	9.9 (3)	105	30.5 (32)	12.4 (13)	57.1 (60)
Village Baretta	5.49	115	71	29.6 (21)	22.5 (16)	47.9 (34)	44	34.1 (15)	27.3 (12)	38.6 (17)
Rorki	7.02	116	59	52.5 (31)	17.2 (10)	30.5 (18)	57	42.1 (24)	8.8 (5)	49.1 (28)
Saideke	8.2	74	38	52.6 (20)	13.2 (5)	34.2 (13)	36	50.0 (18)	8.3 (3)	41.7 (15)
Khara	9.4	191	131	80.1 (106)	13.7 (18)	5.4 (7)	60	46.7 (28)	25.0 (15)	28.3 (17)

* Numbers in brackets indicate number of cases

TABLE 3

Factors Modifying Toxicity of Fluorides

	Village	Mean F^- content of water (ppm)	Incidence of skeletal fluorosis in adult males* (percent positive)	observations
1.	Kooriwara	2.25	40.0 %	Statistically similar water fluoride concentrations, but marked difference in skeletal fluorosis.
	Gurney Kalan	2.45	19.6 %	
2.	Bajakhana	5.09	46.9 %	-same-
	Village Baretta	5.49	29.6 %	
3.	Rajia	5.20	52.2 %	-same-
	Village Baretta	5.49	29.6 %	
4.	Kooriwara	2.25	40.0 %	Water fluoride is much lower in village Kooriwara than in village Baretta. The skeletal fluorosis incidence is higher although the difference is not statistically significant.
	Village Baretta	5.49	29.6 %	
5.	Rajia	5.20	52.6 %	Skeletal fluorosis in these two villages in almost the same, but in village Saideke fluoride content of drinking water is much higher.
	Saideke	8.26	56.2 %	

The underlined villages show a lower incidence of skeletal fluorosis compared to corresponding village.

* Only males have been taken into consideration because of the limited duration of the adult females' residence. The latter migrate to other villages after marriage.

TABLE 4

Influence of Age and Sex on Development of Skeletal Fluorosis*

Age group (years)	ADULT MALES			ADULT FEMALES		
	Number of X-rays	positive cases %	negative cases %	Number examined	positive cases %	negative cases %
21-30	250	15.6 (39)	72.2 (178)	184	14.1 (26)	71.2 (131)
30-40	216	48.2 (106)	29.2 (65)	150	32.0 (48)	58.6 (85)
41-50	151	65.5 (99)	16.5 (25)	120	38.3 (46)	44.1 (53)
51-60	101	69.3 (70)	17.8 (18)	60	41.7 (25)	48.3 (29)
61-70	50	60.0 (30)	24.0 (12)	15	40.0 (6)	40.0 (6)
71 and above	16	75.0 (12)	12.5 (2)	4	50.0 (2)	50.0 (2)

*Total cases: 1848 (males 1315; females 533).

TABLE 5

Skeletal Fluorosis and Age

Village	Mean water fluoride (ppm)	Incidence in Adult Males		
		(> 21 yrs of age % affected)	21-30 yrs age group %	31-40 yrs age group %
Mandi Baretta	0.73	2.6	-	-
Kooriwara	2.25	40.0	8.3	28.6
Gurneykala	2.44	19.6	-	12.5
Ganza Bhanula	4.3	25.0	-	7.7
Baja Khana	5.02	46.6	21.2	60.7
Rajia	5.2	52.2	18.4	49.7
Village Baretta	5.49	29.6	7.9	22.2
Rorki	7.02	52.5	16.7	47.9
Saideke	8.26	55.2	28.6	55.5
Khara	9.4	80.9	60.0	83.7

ished (Table 6). The village of Khara where drinking water contained 9.4 ppm fluoride was the most hyperendemic village of the study. Its residents were the best nourished, whereas in the village of Saideke where the population was poorly nourished and drinking water fluoride was 8.26 ppm, only 55.2% of adult males were affected.

Similar conclusions are warranted from Table 7 which shows no difference in the incidence of skeletal fluorosis between male adults who are financially well to do and those residing in the poorer sections of the town.

4. Physical Stress: Skeletal fluorosis (Table 1) is more common among males who are accustomed to hard work, a most important factor. The exostoses were seen at the sites of muscular exertion. The site of the lesion in 70% of the neurological cases is in the vicinity of the cervical region. This is not unexpected because Indian farmers are required to do digging, ploughing with elementary ploughs and to carry heavy loads on their heads, the kind of work which is likely to put maximum stress on the cervical spine.

The factor of physical stress seems to be mediated by the development of new fluorotic bones at affected sites leading to exostosis formation which may compress the spinal cord and nerve roots.

The lower incidence of skeletal fluorosis in females than in males which has been reported earlier was also seen in the current study. However, this difference in sex ratio was not observed in the poorer residents of villages like Baja Khana, Rorki and Saideke (Table 7). In these villages, the females in the poorer sections have to do hard labor which accounts for an incidence equal to that in males. Similarly in Mandi Baretta where the population is made up of sedentary workers, both sexes were also equally affected. Therefore it seems that sex itself probably plays no role but that the factor of physical exertion is responsible for the sex ratio observed in this disease.

5. Chemical Constituents of Water: From the current study one finds that the level of total hardness, magnesium hardness and chlorides in drinking water was lower in the villages with a higher incidence of skeletal fluorosis (Table 3). Of these, magnesium seems particularly important. Lately the importance of total alkalinity has been pointed out by the Nagpur Group of Public Health workers. A careful study of chemical constituents of waters of the villages given in Table 8 reveals that the villages with a higher incidence of skeletal fluorosis (as compared to a corresponding village) have a lower total and magnesium hardness, less chloride and total solids in their waters and greater alkalinity.

6. Trauma: Trauma itself, although in no way responsible for the development of skeletal fluorosis, did play a role in the development of advanced invalidism. In seven cases, it was either the precipitating or aggravating factor. This may be of importance in that the bone formed under the influence of fluorides is considered to be abnormal and is not like normal bone.

TABLE 6

Nutrient Intake

Village	protein (gm)	fat (gm)	Carbohydrate (gm)	Calories	Calcium (gm)	Phosphorus (gm)	Iron (mg)	Vit. A. International Unit	Vit. B. (mg)	Riboflavin (mg)	Nicotinic Acid (mg)	Ascorbic * Acid (mg)
Mandi Baretta	56.06	71.0	366	2852	0.6	1.6	46.1	3632	1.86	1.56	15.15	35.5
Kooriwara	64.6	68.1	525.9	3166	1.08	2.6	64.7	6792	2.83	1.60	18.2	49.6
Gurney Kalan	87.06	69.25	590.4	3430	1.16	2.6	81.43	4547	2.92	2.15	21.45	43.4
Ganza Dhanaula	92.6	75.5	717.7	3495	1.14	2.8	98.16	5867	3.31	1.85	19.0	61.1
BajakKhana	96.5	74.6	650.2	3542	1.1	2.9	83.59	6770	3.51	1.88	25.13	52.02
Rajia	92.8	97.31	680.6	3635	1.4	2.5	94.2	7069	2.57	2.15	31.45	43.4
Village Baretta	96.9	66.6	592.5	3378	1.2	2.8	83.8	3040	3.07	2.26	25.16	51.4
Rorki	90.5	68.23	566.9	3290	1.13	2.6	69.8	5056	2.78	1.83	21.43	45.73
Saideke	87.0	56.41	493.2	2829	9.4	2.7	74.9	3607	3.06	1.72	24.75	32.3
Khara	109.8	77.16	568.2	3709	1.7	3.7	132.0	4589	3.04	2.63	29.03	57.5

*excluding Vit. C from seasonal vegetable like "Sag"

TABLE 7

Skeletal Fluorosis and Economic Status

Village	Type of population	Daily caloric intake/Unit	Sex	Number examined	% nutrionally deficient	Skeletal fluorosis %
Baja Khana	Jatsikh (prosperous)	4209	Male	85	7.0 (6)	47.0
			Female	112	11.6 (13)	31.3
	Schedule caste (poor)	3292	Male	60	18.7 (11)	47.6
			Female	94	25.5 (24)	56.2
Rorki	Jatsikh (prosperous)	3757	Male	70	5.7 (4)	52.3
			Female	109	8.2 (9)	38.5
	Rai sikh	2827	Male	20	20.0 (4)	53.3
			Female	38	23.7 (9)	50.0

Complications of Skeletal Fluorosis

In 74 cases the neurological deficit of the radiculomyelopathy pattern was observed. No neurological involvement was found in an additional eighteen cases of crippling fluorosis. About 2/3 of the complicated cases were males, all of whom had been drinking water with an average fluoride concentration of 8.9 ppm. The mean age was almost the same for both these groups namely about 56 years. None of these individuals had led a sedentary life prior to their invalidism. Among the neurological cases, in about 70% of the cases with deficit lesions, the latter were located in the cervical region. In seven cases this advanced stage was either precipitated or aggravated by trauma of a trivial nature.

Representative Dietary Surveys

Forty families (4-6 from each village) were questioned about daily dietary intake. The results are tabulated in Table 6.

The various dietary constituents i.e. protein, fats, carbohydrates, vitamins, etc. do not show a marked deficiency in the diet. The caloric intake of these Punjabi villagers is quite adequate except in Mandi Baretta where the population consisted of sedentary shopkeepers.

Most of the calories of a Punjabi diet come from carbohydrates, the chief source of which are cereals and pulses and Gur. An average diet based on data of the 40 families surveyed is presented in Table 9.

TABLE 8

Chemical Constituents of Drinking Water Sources

Village		Fluoride	Total hardness	Calcium hardness	Magnesium hardness	Alkalinity	Chlorides	Phosphorus	Sulfur	Total solid
1. Mandi Baretta	M*	0.73	441.0	90	351	436	198	0.26	42.7	1822
	R	0.37-1.37	400-488	66-112	308-376	380-500	150-235	150-235	29.8-55.1	1640-2110
	SD	0.54	44.37	23.0	36.3	60.2	44.5	0.04	10.4	360
2. Kooriwara	M	2.25	182	70	112	335	10	0.05	23.6	510
3. Gurney Kalan	M	2.44	206	37	169	536	526	0.27	84.99	1990
	R	0.3-7.0	40-734	20-66	20-570	825-865	190-1710	0.5-0.75	16-275	980-3830
	SD	1.1	70.9	24.9	95.7	170.6	276.5	0.20	51.9	662
4. Ganza Dhanaula	M	4.3	155.3	59.1	97.9	510.6	84.4	0.34	58.2	1027
	R	1.0-13.5	28-390	16-128	28-260	285-800	10-208	0.06-0.73	5.8-135.8	161-2580
	SD	1.0	59.7	23.7	36.3	120.8	39.5	0.12	32.5	380.0
5. Baja Khana	M	5.09	140.5	37.3	93.6	834	365	0.33	36.7	1219
	R	0.2-16.6	14-374	8-180	6.522	80-1390	24-950	0.05-0.61	7.7-141.1	780-1751
	SD	2.58	157.7	50.2	125.1	114.02	283.3	0.34	29.4	400
6. Rajia	M	5.2	134.6	52.4	83.00	513.8	74.4	-	124.2	921.2
	R	0.5-12.00	10.428	6-164	4-359	350-720	16.225	-	49.4-274	250-1830
	SD	2.10	82.0	28.1	20.7	120.7	38.9	-	23.7	350.0
7. Village Baretta	M	5.49	280-7	87.7	179.5	578.2	553.6	0-29	68.1	1443
	R	0.37-12.5	26-930	60-112	20-850	380-1275	135-2350	0.1-0.85	1.9-513.6	873-2310
	SD	2.58	157.7	50.2	125.1	414.2	283.3	0.34	73.8	400
8. Rorki	M	7.02	386	77.7	309.1	352	231.5	0.18	31.8	1891
	R	1.5-	75-970	22-340	63-630	320-658	80-510	0.010-.65	5.7-135.0	450-4550
	SD	3.52	197.7	57.2	153.01	112.6	92.5	0.09	25.7	828
9. Saideke	M	8.26	170.4	31.95	140.7	665.2	170.34	-	132.5	2313
	R	3.5-170	64-290	10-270	36-272	540-1150	65.250		78.7-246	990-3500
	SD	2.8	67.3	25.7	78.4	170.3	98.7		49.3	560-5
10. Khara	M	9.4	75.5	22.7	49.51	910.6	105	0.13	32.8	1498
	R	3.0-14.0	16.125	50-126	80-188	110.1200	15.255	0.05-0.20	5.4-61.5	760-2120
	SD	1.8	34.2	17.3	34.9	164.5	65.7	0.12	14.7	375

* M - Mean R - Range SD - Standard Deviation .

TABLE 9

Composition of an Average Adult Diet

Cereals	Quantity in Gms. (or ml)
Wheat	415
Maize	50
Milk	Limited
Pulses	46
Milk and Milk preparations	220 ml.
Oils and Fats (Ghee)	28
Vegetables Leafy	113
Others	160
Chillies	5-10
Sugar/ Jaggry	138
Common salt	10-20
Meat, fish and eggs	occasional

Two villages one of which was prosperous and the other economically deprived living side by side and consuming similar drinking waters provided an opportunity to study the effect of nutrition on the development of skeletal fluorosis. These two villages are compared in Table 7.

From this table it can be seen that among adult male subjects from the rich and poor villages of Baja Khana and Rorki the incidence of skeletal fluorosis did not differ in spite of a marked nutritional and socio-economic difference. Had nutrition provided any protective effect, it should have been evident in these villages.

However, among females of these two sections a significant difference was found. In the poorer community the incidence of skeletal fluorosis was almost equal to that in males possibly because women of poor communities are obliged to work harder and the factor of physical stress plays its role in them.

Fluoride Content of Food Stuffs

Forty samples of common food commodities like cereals and pulses obtained from an endemic village and 3 samples of common salt were analyzed as shown in Table 10.

The major part of an average diet of the surveyed population is made up of cereals, pulses, milk and its products. On the basis of this diet, an average villager in an endemic area has been calculated to be getting about 1.12 to 1.64 mgm of fluoride daily from these uncooked foods which is a small amount compared to that ingested from water. Other common food materials like vegetables (leafy and others) milk, tea, alcohol etc. need to be analyzed.

TABLE 10

Fluoride in Food

Food Material Analyzed	No. of samples analyzed	Fluoride content (ppm netweight) (mean)	(Range)
Wheat (whole)	10	1.53	0.1-2.8
Maize	6	3.19	1.95-2.5
Millet	4	2.93	1.93-3.5
Bengal Gram	6	2.00	0.48-3.24
Green Gram	2	0.69	0.56-0.72
Lentil	4	2.55	2.1-3.6
Mash	3	2.39	1.0-4.4
Common salt	3	20.0	15.3-24.3

Fluoride Content of Soil of Endemic Villages

Fifteen soil samples collected from the outskirts of the villages were estimated to contain 176-389 ppm fluoride with an average value of 241.2 ppm.

Urinary Fluoride Excretion

The pattern of urinary fluoride excretion was studied in 68 cases by collecting 24 hour urine samples in 3 villages of the current study and analyzing them for fluoride along with the drinking waters of these subjects (Table 11).

TABLE 11

Urinary Fluoride Excretion in Persons Living in Endemic Villages

Village	F^- Content Drinking Water (ppm)	Urinary Fluoride concentration (ppm)	24 hours Urinary fluoride (mg per day)	Urinary fluoride per gram of creatinine in urine (mg)
Baja Khana (20)*	11.05 (2-16.6)	10.49 (2.75-25.75)	5.95 (1.9-11.33)	12.35 (3.42-29.50)
Ganzadhanaula (28)*	4.5 (1.1-11.1)	4.31 (0.25-15.0)	3.72 (0.19-13.8)	5.3 (0.32-12.3)
Mandi Baretta (20)*	0.73 (0.3-1.38)	2.13 (0.25-4.23)	1.6 (0.15-2.8)	2.35 (0.35-4.67)

* number of samples analyzed.

In two villages of Baja Khana and Ganga Dhnaula the fluoride concentration of urine (in ppm) correlated very well with drinking water fluoride content (in ppm) whereas in Mandi Baretta the urine fluoride concentration was higher. The urinary fluoride per mg of creatinine also gave comparable readings.

Calcium Balance Studies

Calcium balance studies were completed in 25 hospitalized cases of endemic fluorosis. In all of them, uniform results showed a retention of calcium ion. The urinary and faecal calcium excretion was decreased in cases of fluorosis.

Discussion

Dr. Moser: You stated that in areas where the calcium content of water is high, the rate of skeletal fluorosis is low and that in endemic areas the calcium and magnesium levels of natural water are low. Would it be possible to add calcium and magnesium to the water to retard the absorption of fluoride? - You mentioned that the water has a very high fluoride content - up to 40 ppm. We have developed methods to precipitate excess fluoride out of the water.

Dr. Jolly: The patients with skeletal fluorosis do not have a deficiency of calcium and magnesium in their diets. Adding these minerals to the water might not help. - Defluorination of water supplies is now in progress in some areas of India, but it is a very expensive operation.

Dr. Mohamed: If fluorosis is more common in the male than in the female, records should be kept to determine whether the disease is hereditary, or handed down genetically. - Is mongolism prevalent in the endemic areas? In some areas of Texas, U.S.A. where the water has a high fluoride content (2-8 ppm) the incidence of mongolism is high. Fluoride has been proven to produce chromosomal defects. This abnormality can conceivably be handed down as a mutagenic agent.

Dr. Jolly: Our records show that children who move to other areas away from their families who are residing in endemic areas do not develop as much skeletal fluorosis as those who remain with their parents. - We have not found a high prevalence of mongolism in our series.

Dr. Franke: I am familiar with the skeletal changes described by Dr. Jolly. We encountered them in industrial fluorosis. As to Dr. Mohamed's comment, we have seen cases in which genetic changes indicative of a hereditary factor have appeared. - Changes in the spinal canal are not seen in industrial fluorosis, or elsewhere, other than in India. Is this fact related to the type of work in which those people are engaged?

Dr. Jolly: Most of the work involves farm labor; heavy loads are carried on farmers' heads.

Dr. Cook: Has Dr. Jolly noted any differences in skeletal and nonskeletal fluorosis? - What is the degree of kidney involvement in each type?

Dr. Jolly: There is no difference in kidney disturbances between skeletal and nonskeletal fluorosis. - Only two postmortems in our series showed kidney failure.

Dr. Sinclair: Prof. Jolly, how do you explain the increased calcium balance and decreased serum calcium in your cases?

Dr. Jolly: As yet we have no explanation for it.

A REVIEW OF THE EFFECT OF FLUORIDE ION ON ADENYL CYCLASE

by

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SUMMARY: Since its discovery and identification in 1957, the compound adenosine 3',5' cyclic monophosphate (C-AMP) has played an ever increasing role in the elucidation of a multitude of biochemical pathways. The enzyme adenyl cyclase catalyzes the conversion of adenosine triphosphate (ATP) to C-AMP and has been a primary area of research interest.

One of the unifying points in the vast amount of research has been the positive response of adenyl cyclase to the addition of low concentrations of fluoride. A stimulation effect for the addition of fluoride appears to be almost universal, at least for the particle-bound enzyme, but certain variations exist. Current investigations are underway to study the effect of fluoride. It appears that the mechanism of the stimulation of adenyl cyclase activity by fluoride differs from that of a variety of hormonally-active organic compounds.

I. Introduction and History

Since the discovery and identification of 3',5' cyclic adenosine monophosphate (C-AMP) by Sutherland, et al. (1) in 1957, the biochemical and related literature has contained an ever increasing amount of material that has tied C-AMP and related compounds to a wide variety of enzyme and hormone systems. One of the basic components of this unfolding story has been the role played by the enzyme adenyl cyclase. This enzyme catalyzes the formation of C-AMP from its parent compound adenosine triphosphate (ATP) according to the reaction:

$$ATP + H_2O \xrightarrow[\text{Cyclase}]{\text{Adenyl}} \text{C-AMP} + \text{Pyrophosphate} \quad (1)$$

Adenyl cyclase is found in a wide variety of organisms ranging from bacteria to a number of mammalian tissues. It is particulate in nature, i.e. membrane-bound in its native state in higher animals and, in general, the highest activities are associated with the mitochondrial and microsomal fractions of the cell.

The unusual feature of the adenyl cyclase story, however, is not the chemistry involved in the formation of C-AMP but rather the unique role played

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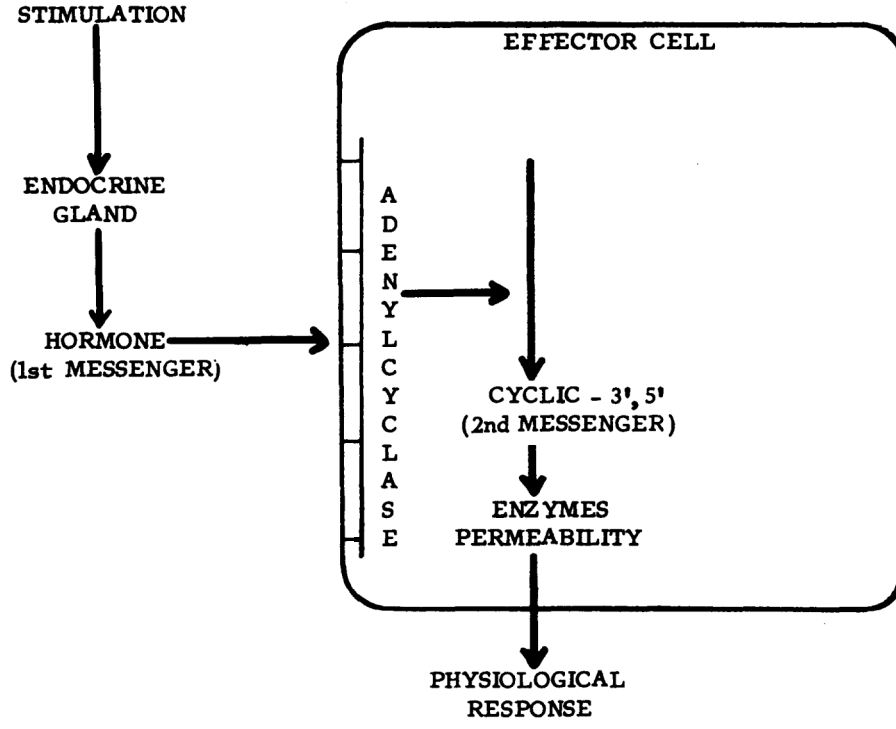
by C-AMP and certain of its derivatives and analogues as mediators of hormonal action. By its response to hormones adenyl cyclase influences a wide variety of reactions through C-AMP. The response of adenyl cyclase to a given hormone appears to be related to the tissue source of the enzyme with epinephrin, norepinephrin, glucagon or thyroid hormone stimulating adenyl cyclase, depending on the tissue from which the enzyme was obtained.

The overall mechanism associated with the hormonal response mechanism is shown in Figure 1. Within the scheme, the first messenger (hormone) is re-

Figure 1



leased in response to stimulation and is carried to the effector cell. The effector cell is differentiated on the basis of whether or not the adenyl cyclase of that cell responds to the stimulation of the particular hormone. Obviously the target cells contain adenyl cyclase with the proper chemistry for combining with the particular hormone and thus the cyclase enzyme is activated. Once activation of adenyl cyclase has taken place, increased synthesis of C-AMP (second messenger) results in a wide variety of changes that may occur within the cell, e.g. increased enzyme activity and change in membrane permeability. These

changes at the molecular level lead to a physiological response that rapidly becomes apparent. The first messenger (hormone) builds into the system the specificity needed while the second messenger (C-AMP) stimulates one or more of a possible multitude of reactions. *

Recent evidence indicates that certain C-AMP derivatives and analogues may play important roles in the second messenger concept. Various other cyclic nucleotides, e. g. cyclic guanidine monophosphate as well as cyclic-AMP derivatives, e. g. N, 0' dibutyryl cyclic adenosine monophosphate have been shown to have marked physiological activity in certain tissues. However, the chemical mechanism of the formation and their action is not as well established as for C-AMP.

The overall picture of the action of C-AMP is extremely complex and confusing. Perhaps this is the reason that many investigations deal with the response of adenyl cyclase to the fluoride ion. Whereas adenyl cyclase exhibits a widely varying response to hormones depending upon the source of the enzyme, addition of fluoride stimulates the particulate-bound enzyme in almost all cases.

II. Effect of Fluoride Ion on Adenyl Cyclase

One general aspect of the fluoride response of particulate adenyl cyclase is the lack of stimulation of whole cells or intact tissues. Direct stimulation by fluoride of adenyl cyclase has not been demonstrated in intact tissue of cells except for adipose cells. For example, the fluorine ion has no effect when it is added to washed erythrocytes; following hemolysis, however, fluoride will produce its characteristic response. A similar situation exists in the brain. A concentration of fluoride ion in brain homogenates that stimulates maximally is without effect on intact brain slices.

The magnitude of the fluoride stimulation may range from an increase of 25% (2) to 900% (3) to an inhibition in certain bacterial adenyl cyclases. The average stimulation of adenyl cyclase activity by fluoride is a three to fourfold increase in activity. This response also varies with the concentration of fluoride. Most investigators have used a fluoride concentration of 10mM (400 ppm).

In their early work Rall and Sutherland (4) reported the effect of fluoride on adenyl cyclase from liver. They pointed out that the production of C-AMP was reduced to approximately one-third of the value found when fluoride was not added to the incubation mixture. Certain facts concerning the mechanism of this stimulation have now become apparent. For example, several similarities exist in the stimulation of adenyl cyclase by hormones and by fluoride. Both

*Dr. Sutherland was awarded the 1971 Nobel prize for medicine for his discovery and description of the second messenger concept.

exhibit similar pH optima. However, among important differences: Hormonal activation is reversible whereas fluoride activation is not. The stimulating effect of a given hormone may be washed or dialyzed away whereas the stimulating effect of the enzyme by fluoride is not removed by dialysis or by repeated washing (5-7). Adenyl cyclase, derived from the pineal gland of rats, responds to fluoride and to norepinephrin as described above. Furthermore the use of β-adrenergic blocking agents prevents only the norepinephrin stimulation, but does not affect the fluoride response. Interestingly, freezing and thawing, as well as phentolamine (an alpha-adrenergic blocking agent) increase the response of adenyl cyclase to both fluoride and norepinephrin.

Chlorpromazine also blocks the hormone stimulation of the cyclase enzyme from a number of tissues without affecting basal levels of activity. Stimulation of kidney adenyl cyclase by parathyroid hormone is the sole exception and is unaffected. A concentration of chlorpromazine greater than 3.0×10^{-4}M increased the activation of the enzyme by fluoride. On the basis of comparison a number of other agents with a somewhat similar activity, e.g. thymol, the surface properties of chlorpromazine have been held to be the mediating factor.

Comparison with other stimulating agents also suggests that the stimulating mechanism of fluoride is unique. Cyclase enzyme from the cerebral cortex of rats is enhanced by fluoride; this effect is dependent upon temperature and upon the concentration of the fluorine ion (5). The enzyme treated with fluoride is more stable to heat denaturation as well as to dialysis than the native enzyme. Similar to cyclase from many other sources, the enzyme of the cerebral cortex is stimulated by detergents. If the enzyme is treated with a detergent (Triton X-100) no further stimulation by fluoride takes place, but if fluoride is added to the enzyme first, the activity is subsequently further stimulated by treatment with detergent.

There appears to be an interaction between fluoride and certain inorganic stimulants of adenyl cyclase, e.g. Mg^{++}. In guinea pig heart preparations (7) and in cyclase from several other sources, magnesium ion stimulates the enzyme in the absence of fluoride. However this effect is not a maximal stimulation since the addition of fluoride to Mg^{++} treated samples will further enhance the enzyme activity. Calcium ion behaves in an opposite manner: it inhibits adenyl cyclase regardless of whether or not fluoride is present. There is some evidence that fluoride may become bound to the enzyme as the magnesium complex (8).

One additional possibility for the stimulation, which was part of the original idea behind the addition of fluoride to the incubation mixture, is the inhibition of ATP ase (9). This approach may be functional in some cases but several experimental procedures indicate that this mechanism does not explain the fluoride stimulation, e.g. addition of excess ATP does not increase C-AMP yield in the absence of fluoride.

On the basis of the above observations, certain theories have been devel-

oped to describe the action of fluoride and other stimulating agents on adenyl cyclase. The available data suggest two distinct types of mechanism. One involves the solubilization phenomena associated with membrane-bound enzymes, th s the effect of certain agents such as chlorpromazine may relate to their ability to change confirmation, surface activity or related properties.

An alternate approach would indicate that the activities may relate to the removal of an inhibitor protein. It has been suggested, at least for certain types of adenyl cyclase (rat parotid glands), that the activity of the enzyme is controlled through a specific protein (or proteins) which couples with the enzyme and inhibits its activity. It is believed that stimulating hormones act by linking to a site on the inhibition protein and thus reversibly remove the inhibition. Fluoride might then act by counteracting the inhibition by a mechanism not readily reversible (10). This mechanism might involve complete removal and denaturation of the inhibition protein in such a manner that recombination with cyclase would be impossible.

III. Factors that Influence the Fluoride Effect

To date, it has not been possible to irrefragably assign a reaction sequence to the above-described mechanisms. One of the difficulties that arises in the determination of an overall mechanism, if indeed one exists, is the wide variety of factors which influence the response of adenyl cyclase to fluoride. Such variables include the source tissue, the cellular fraction, the effect of freezing and thawing, the effect of detergents, variations with temperature, presence of other inorganic ions, purity of the cyclase enzyme and the influence of temperature on assay. Thus there are many similarities as well as many variations between adenyl cyclases from various sources.

A. Source of Tissue

Species differences are recognized in the area of comparative biochemistry, but explanations of these differences vary. At the molecular level, variations in chemical structure, e.g. amino acid sequence in the protein chain, are common among enzymes. Unfortunately, it has not been possible to isolate pure adenyl cyclase from a variety of sources which would permit one to make valid comparisons. However, comparison of the fluoride responses of cyclase from a number of different species have been made. In order to minimize variables, similar tissue is generally used for these comparisons. Schmidt et al. (11) studied cyclase from erythrocyte ghosts* of cats, rats, dogs, mice and humans. All cells were stimulated by fluoride and norepinephrin. The response of rat and mouse red cells was enhanced compared to that of other animals which indicated that the cyclase activity was not related to an active sodium pump. Additional experiments have also demonstrated that the fluoride effect upon red cell ghost is not related to increased ATP availability (12).

*Cells that have had their hemoglobin removed and leave behind the particulate cell membrane.

Furthermore, variations of cell types within a given tissue point up important differences. Thus, liver tissue has been shown to differentiate between glucagon and isoproterenol responses. The glucagon-sensitive enzyme is found in the parenchymal cells whereas the isoproterenol responding enzyme is present in the reticulo-endothelial cell as well as in the parenchymal cell. Fluoride-stimulated enzyme is found in both (13).

B. Age of Source Animal

An alternate method of comparing adenyl cyclase activity is to use the enzyme from animals of the same species at different ages. Rat brain has been shown to contain elevated levels of C-AMP for three to six days postpartum. The ability of norepinephrin to stimulate the enzyme follows the same time sequence. In the absence of fluoride, adenyl cyclase levels increased until approximately day seven and then decreased. In the presence of fluoride, however, adenyl cyclase activity continued to increase. These data suggest that fluoride removes an inhibitor from the cyclase which may be partially or even totally absent at birth (11).

Comparison of adenyl cyclase in fat cell homogenates from rats of varying ages shows an interesting fluoride effect (1). Comparisons of young (5-6 weeks), middle aged (10-12 weeks) and old (18-24 weeks) rats revealed that both fluoride and norepinephrin stimulation decreases markedly with advancing age. In the middle-aged and young rats, the fluoride stimulation was approximately 50% of that induced by norepinephrin whereas in old animals the stimulation was almost equal for the two agents. In the intact cells, the norepinephrin response was similar to that from fluoride.

Variation in response to fluoride according to age is also demonstrated by rabbit aorta (14). Both thoracic and abdominal aorta homogenates respond to addition of fluoride, the abdominal response being much higher. The lowered response of the thoracic aorta remains constant with increasing age as do the basal levels of the enzyme. In contrast, the abdominal aorta shows an increase in fluoride stimulation with advancing age whereas the basal levels remain unchanged.

Another method of comparing enzyme activities from the same tissue is to use mutant or tumor cells. Hela cells, 3Tb fibroblast, Chang's liver cells, rat liver and cat liver have been assayed for their adenyl cyclase contents (15). All were stimulated by fluoride; however, the extent of the stimulation depended upon the manner in which the cells were incubated and grown. This finding casts doubt on the validity of comparing data from different laboratories, particularly for cultured cells.

C. Cellular Fraction

The question of the influence of cellular location on the fluoride response

of adenyl cyclase must be viewed in the light of the variations already mentioned. The fluoride response has been measured in a wide variety of cellular preparations ranging from tissue slices to the cytoplasmic fraction. Whereas no direct action has been measured in tissue slices, the effect of fluoride added to thyroid slices and the similarity to the effect of C-AMP addition on glucose oxidation has been noted (16).

Generally speaking adenyl cyclase has been found to be associated with the membrane fraction from almost all cell organelles if allowance is made for species variation. Some enzymes do not follow this pattern. One report (17) relates to a plasma membrane adenyl cyclase, with unique properties in several areas. This enzyme was inhibited by the presence of Na^+ and when fluoride (5mM) was added approximately a 60% inhibition of the enzyme activity took place.

Another reported variation is associated with a soluble (cytoplasmic) adenyl cyclase. As is often the case, certain bacteria appear to have nonmembrane bound adenyl cyclase. The enzyme from these sources does not entirely duplicate the particulate-derived enzyme. A purified enzyme from B. liquefaciens (18) has been shown to require pyruvate as well as Mg^{++} but no pyruvate. This enzyme was markedly inhibited by the addition of fluoride and sodium pyrophosphate. The addition of fluoride in a concentration of 5 mM (200 ppm) caused an 80% inhibition of activity in the purified enzyme. This inhibition was not accounted for by the possible binding of Mg^{++} above that which is required for optimal activity because addition of Mg^{++} beyond the concentration necessary to give the maximum fluoride complex did not reverse the fluoride inhibition. Other studies indicate many differences in the bacterial enzyme (19).

D. Effect of pH

Only a few investigations have fully explored the possible relationship of pH to the effect of fluoride upon adenyl cyclase. Many enzyme preparations require Mg^{++} for maximal activity which, depending upon the pH of the solution, may exist as the complex of fluoride and, additionally, may bind the substrates as the Mg-ATP complex. The relative contribution of these species would obviously depend upon the pH of the media.

The particulate-derived enzyme has a pH optimum of approximately 7.5 (20). A survey of adenyl cyclase from preparation of dog tissue including brain, spleen, muscle, heart, liver and lungs revealed a general maximum in the range of 7.2 to 8.2 pH units (21). Of these preparations, the heart derived enzyme appeared to have a higher pH optimum in the absence of fluoride than in its presence.

Salmon testis adenyl cyclase has a very sharp pH maximum of 7.5 in phosphate buffer (22). These observations were made in the presence of the optimum concentrations of fluoride (7.0 mM) and Mg^{++} (10 mM).

E. Fluoride Ion Concentration

Most investigators have used a fluoride ion concentration in the vicinity of 10 mM if testing only for a fluoride response. The choice is a good one as many sources of adenyl cyclase yield an enzyme that has maximal activity in the presence of 10 mM fluoride. Rat cerebral cortex shows a maximal effect for fluoride concentrations in the range 4 mM to 10 mM (4). Enzyme from salmon testis has a broad maximum at 7.0 mM fluoride and shows a slight decrease as the concentration is raised to 22 mM (22). Cyclase from dog heart sarcoplasmic reticulum shows a similar response curve (23). Rat kidney enzyme has a broad response maximum between 10 and 30 mM (5).

The enzyme from rat fat cells has been shown to have a maximum response to 3.0 mM fluoride with a 15% loss in activity at 1.5 or 7.7 mM. However the activity was not completely lost in the presence of 80 mM fluoride. This effect was not related to sodium ion as a similar concentration of sodium chloride did not produce the same results (24). Heart adenyl cyclase from hypothyroid cats exhibited a routine response to fluoride ion concentration (maxima between 4.0 and 10 mM) whereas the overall activity of the hypothyroid animals was considerably less than that of the euthyroid animals (25).

To test the effect of fluoride on developing rat brains, Pastan et al. (10) used three concentrations of fluoride namely 2.5, 5.0 and 10.0 mM. In brain homogenates from newborn and five-day-old animals, the response to fluoride was approximately equal for all concentrations (only slight stimulation took place). At nine days, a clear stimulation was observed with the 5.0 mM concentration being the most effective. From the ninth to the twenty-third day the basal levels fell rather sharply; however, stimulation by fluoride as a percentage of basal level, continued to increase. At day twenty-three the order of stimulation was clearly 2.5, 5.0 and 10.0 mM.

F. Miscellaneous Factors

A large number of additional parameters of adenyl cyclase have been investigated. Since these observations have been reported for the enzyme from only one or perhaps two sources, it is difficult to assess their general applicability. Many of the parameters investigated are unique to adenyl cyclase.

Freezing and thawing of membrane preparations of rat cerebral cortex is an example of an unusual stimulating factor associated with adenyl cyclase (4). This treatment gives rise to a twofold increase in enzyme activity. Weiss (5) has found dissimilar results with respect to rat pineal gland cyclase. If this material is frozen or allowed to stand at 30°C, the response to fluoride and norepinehrin is reduced by similar degrees and at the same rate. The pH optimum is also the same. However, on the basis of additional observations it was concluded that two sites of action exist.

The treatment of membrane preparations of adenyl cyclase with detergents elicits a response that elucidates the overall mechanism of fluoride stimulation. Treatment of cat heart enzyme with the detergent Lubrol-PX gives a soluble preparation that is responsive to fluoride but unresponsive to norepinephrin and to glucagon which stimulate preparations not treated by the detergent (26). These observations may relate to the findings in bacterial adenyl cyclase where the soluble enzyme does not always respond to the normal hormonal stimulation.

The recent report (27) of an interrelationship between hormonal stimulation and phospholipid content of a solubilized adenyl cyclase from cat heart may point the way to an overall understanding of the stimulation by both hormones and fluoride. Treatment with detergent (Triton-X) in the case of cyclase from rat cerebral cortex induces direct stimulation above that found for fluoride as compared to the basal levels of the enzyme (4). Liver plasma membrane preparations show a somewhat similar effect (22). In cerebral cortex tissue the stimulation may involve an activation process. The time interval following addition of fluoride may be important along with its concentration.

The effect of the temperature of the assay adds evidence to the activation theory. Increase in temperature of the assay media below the denaturation temperature of the enzyme decreases the time necessary for a maximum stimulation by a given concentration of fluoride. This presumably results from an increase in the rate of activating. However, the rate of stimulation is not only increased but the overall stimulation is elevated at higher temperatures (4).

Whereas fluoride stimulation is widespread, a few compounds can reverse the process at least to a certain degree. For example, rabbit renal cortex is stimulated by addition of fluoride; however, when Ca^{++} is added the stimulation decreases by 35% (28). Similar effects have been noted in liver (29). In guinea pig heart, Ca^{++} completely blocks the enhancement of the enzyme's activity by fluoride (7). Sarcoplasmic reticulum of dog heart actively concentrates Ca^{++} which the addition of fluoride lowers while stimulation of adenyl cyclase takes place. Epinephrin also stimulates the enzyme but is without effect on the uptake of Ca^{++} (30). Adenyl cyclase from thyroid tissue has a positive response to thyroid stimulating hormone (TSH) and fluoride. This response is reduced by the addition of calcium. Ouabain, on the other hand, abolishes the TSH effect, but enhancement by fluoride is not affected.

Rat liver adenyl cyclase has been reported to be inhibited by adenosine and certain adenine nucleotides which also decrease fluoride stimulation (8). Theophyllin has been shown to inhibit the enzyme from rat red cell ghost but does not influence the effect of fluoride (31).

Fluoride and many hormones appear to affect adenyl cyclase activity in a related manner. But many differences exist in their action as shown by kinetic studies. This may be due to an increase in Vmax of the enzyme reflecting increased reactivity of the catalytic site (32). Additional Vmax studies (33) indicate the enzyme from a single source may exist as isozymes which would partially explain the complexity of the response.

TABLE 1

Compilation of Some of the Recent Literature on Fluoride Effects on Cyclase

Source	F^- Stimulation	Other Stimulants	Comments
Rat cerebr. cortex 1000xg (5)	5mM (X2.5)	Temp., Freezing, Detergents	F^- not dialyzable from the cyclase
Rat and og liver 2000xg	10mM, (X3)	Freezing Thawing	Freezing and thawing - little effect
New born rat brain 78000xg (11)	5mM, (X1.5)		F^- stimulation may be reversal of inhibition not present at birth
Dog, sheep & calf thyroid slices (16)	3mM	PGE_1	F^- mimicked C-AMP in effect on glucose oxidation
Sheep thyroid slices (35)	40mM	Various nucleotides	No inhibition with high F^- levels
Breast tumor 100,000xg (36)	10mM	Epin.	Epin. stimulated more than F^- plus epin.
Morris hepatoma 100,000xg (37)	10mM	Epin.	
Morris hepatoma 100,000xg (38)	10mM	Epin.	Author points to difference in tumor tissue
Sheep thyroid slices (3)	10-25mM (x9)	TSH, Glucagon	TSH & glucagon stimulation blocked by beta-adrenergic blockers
Rat pineal gland tissue homogenate (39)	10M, (X8)	Norepin. plus hormones	Estradial inhibits F^- stimulation of AC
Sheep & cat brain; beef heart; lung; spleen muscle; pigeon erythrocytes (40)	10mM		F^- effect not determined on all tissue
Bordetella pertussis	10mM	Epin.	F^- had no effect on cyclase from this bacteria
Rat & human mucosa (42)	1mM	All so tested effect of F^- on ATPase	F^- stimulation may be due to inhibition of ATPase
Erythrocytes ghost (12)	(X3)	Norepin.	F^- stimulated more than norepinephrine
Cat heart (25)	4-10mM	Norepin.	F^- stimulates hypothyroid cat less than normal
Rat pineal 100,000xg (6)	0.25-5mM	Epin.	F^- stimulation does not involve ATPase inhibition
Salmon testis (22)	4-10mM (X1.5)	Mg^{++}	Enzyme very labile
Fetal rat calvaria (43)	10mM	Parathyroid hormone	

TABLE 1 (cont.)

Source	F^- Stimulation	Other Stimulants	Comments
Frog erythrocytes (44)	(X3)	Norepin.	75% of that found in absence of F^-
Guinea pig heart 1000xg (8)	8mM	Epin.	F^- activity above that of Mg^{++}, Ca^{++} is inhibitory
Fat cells (rat) (24)	3mM (X10)	Glucose Epin.	Insulin and prostaglandin E_1 were without effect
Cat heart homogenate (45)	10mM (X10)	Epin.	Denervation does not affect C-AMP production nor degradation
Dog heart 1000xg (23)	8mM (X3)	Epin.	First report of cyclase in cardiac sarcoplasmic reticulum
Human platelets (46)	10mM (X20)	Glucagon	
Human platelets (47)	0.7mM (X10)	Prostglandin E_1	Prostglandin E_1 stimulated more than F^- (18x)
Guinea pig skin (48)	10mM		Epin. did not stimulate in absence of F^- PGE_1; glucose, serotonin not active
Rabbit renal cortex (49)	10mM	Parathyroid Ca^{++}	Ca^{++} decreased F^- effect but did not totally remove stimulation
Adrenal tumor 105,000xg (10)	20-30mM (X)	ATCH	F^- removes an inhibitor
Rabbit small intestine (50)	(X5)	PGE_1	Cholera toxin stimulates gut adenyl cyclase about the same as F^-
Erythrocytes (15)	10mM (X3)	Norepin.	Norepin. stimulated mouse and rat but no others
Rat liver 2000xg (9)	10mM (X4)	ADP, AMP Inosine adenosine, IMP	ADP, AMP and adenosine in presence of F^- inhibit 40%, 50% & 60% respectively
Rabbit heart (14)	10mM (X3)	Histamine	Histamine gave no stimulation
Bovine adrenal cortex, 8200xg (51)	10mM (X3)	ACTH	8200xg and 105,000xg pellet and greatest specific activity
Beef thyroid (2)	10mM (X1.25)	Epin. prostaglandin ACTH	Chlorpromazine ($3x10^{-4}$) inhibits Epin. thyro. PGE_1 stimulation. Basal levels uneffected, F^- stimulation inhanced
Bovine & canine thyroid homogenate (52)	10mM	TSH, ACTH	TSH stimulates above F^- stimulated levels
Liver tumor (53)	8mM (X20)	Epin. & Glucagon	F^- stimulation depends on how cells grown

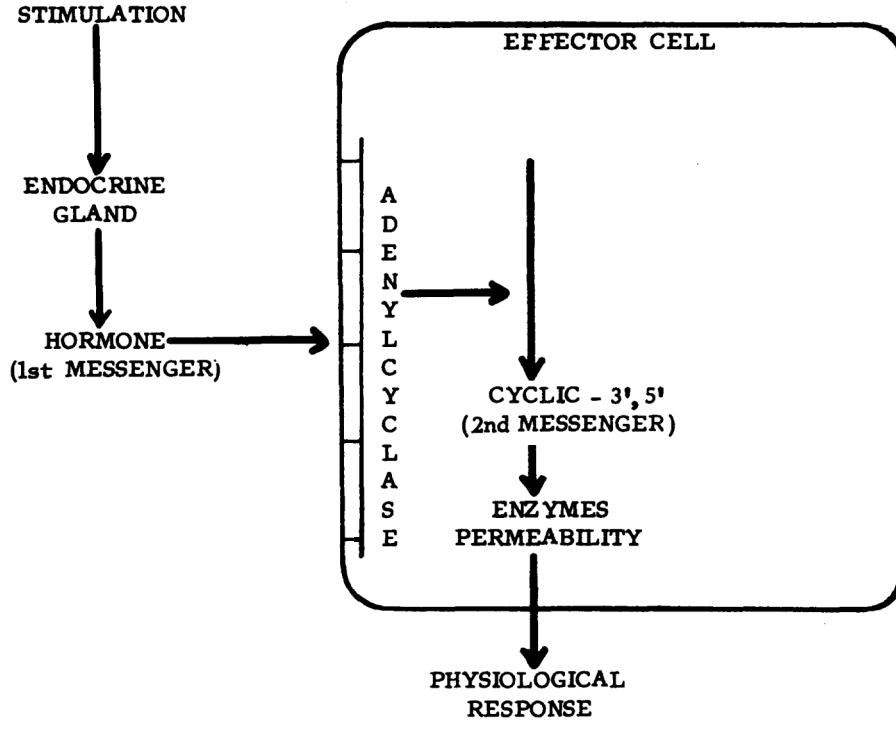
TABLE 1 (cont.)

Source	F^- Stimulation	Other Stimulants	Comments
E. coli (54)	5mM	Pyrophosphate	F^- inhibits as does pyrophosphate
Dog heart sarcoplasma 40,000xg (30)	5mM (X3)	Epin.	Ca^{++} uptake was increased 40% by F^-, not fected by epin. stimulation
Rat parotid gland (7)	10mM (X1.5)	Norepin.	Hormone binds to cyclase releases inhibitor but is reversible
Rat liver (13)	12mM (X4)	Glucagon	F^- stimulated cyclase from both cell types
Rat erythrocyte ghost (31)	10mM (X5)	Norepin., & theophylline	Theophylline inhibits norepin. stimulation, does not affect F^- stimulation
Cat heart homogenate (26)	8mM (X2)	Norepin., glucagon, thyroxin	Lubrol-PX used to solubilize cyclase, F^- stimulation unaffected others didn't stimulate solubile enzyme
Frog erythrocytes (55)	10mM		Factor from clostridial neuraminidose stimulate adenyl cyclase
Rat fat cells (1)	10mM	Norepin.	Adenyl cyclase decreased with age, F^- stimulated but relative values remained same
Bovine thyroid plasma membranes and whole homogenate (56)	10mM (X4)	TSH	TSH stimulated adenyl cyclase from membrane and whole homog., F^- affected both
Rat Kidney 600xg (20)	3-100mM (X3)		Studied properties of enzyme
Rat liver plasma membrane (17)	10mM	Glucagon	F^- inhibits the cyclase activity
Sheep thyroid homogenate (57)	10mM (X5)	Li^+, Na^+, K^+, TSH	F^- stimulates above TSH level Li^+, Na^+ & K^+ alter stimulation somewhat, F^- removes onabain inhibition of adenyl cyclase
Mice adrenal tumors (58)	0.4-40mM	ACTH	ACTH did not stimulate above F^- stimulation
Rat fat cell ghost (59)	10mM	ACTH, Epin.	F^- stimulated maximally, F^- appeared to have different site of action
Beef thyroid slices (60)	3.5-21mM (X4)	TSH	F^- stimulate glucose oxidation but doesn't cause intracellular colloid droplets to form; dibutyl C-AMP does

by C-AMP and certain of its derivatives and analogues as mediators of hormonal action. By its response to hormones adenyl cyclase influences a wide variety of reactions through C-AMP. The response of adenyl cyclase to a given hormone appears to be related to the tissue source of the enzyme with epinephrin, norepinephrin, glucagon or thyroid hormone stimulating adenyl cyclase, depending on the tissue from which the enzyme was obtained.

The overall mechanism associated with the hormonal response mechanism is shown in Figure 1. Within the scheme, the first messenger (hormone) is re-

Figure 1



leased in response to stimulation and is carried to the effector cell. The effector cell is differentiated on the basis of whether or not the adenyl cyclase of that cell responds to the stimulation of the particular hormone. Obviously the target cells contain adenyl cyclase with the proper chemistry for combining with the particular hormone and thus the cyclase enzyme is activated. Once activation of adenyl cyclase has taken place, increased synthesis of C-AMP (second messenger) results in a wide variety of changes that may occur within the cell, e.g. increased enzyme activity and change in membrane permeability. These

TABLE 1

Compilation of Some of the Recent Literature on Fluoride Effects on Cyclase

Source	F^- Stimulation	Other Stimulants	Comments
Rat cerebr. cortex 1000xg (5)	5mM (X2.5)	Temp., Freezing, Detergents	F^- not dialyzable from the cyclase
Rat and og liver 2000xg	10mM, (X3)	Freezing Thawing	Freezing and thawing - little effect
New born rat brain 78000xg (11)	5mM, (X1.5)		F^- stimulation may be reversal of inhibition not present at birth
Dog, sheep & calf thyroid slices (16)	3mM	PGE_1	F^- mimicked C-AMP in effect on glucose oxidation
Sheep thyroid slices (35)	40mM	Various nucleotides	No inhibition with high F^- levels
Breast tumor 100,000xg (36)	10mM	Epin.	Epin. stimulated more than F^- plus epin.
Morris hepatoma 100,000xg (37)	10mM	Epin.	
Morris hepatoma 100,000xg (38)	10mM	Epin.	Author points to difference in tumor tissue
Sheep thyroid slices (3)	10-25mM (x9)	TSH, Glucagon	TSH & glucagon stimulation blocked by beta-adrenergic blockers
Rat pineal gland tissue homogenate (39)	10M, (X8)	Norepin. plus hormones	Estradial inhibits F^- stimulation of AC
Sheep & cat brain; beef heart; lung; spleen muscle; pigeon erythrocytes (40)	10mM		F^- effect not determined on all tissue
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TABLE 1 (cont.)

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Rabbit small intestine (50)	(X5)	PGE_1	Cholera toxin stimulates gut adenyl cyclase about the same as F^-
Erythrocytes (15)	10mM (X3)	Norepin.	Norepin. stimulated mouse and rat but no others
Rat liver 2000xg (9)	10mM (X4)	ADP, AMP Inosine adenosine, IMP	ADP, AMP and adenosine in presence of F^- inhibit 40%, 50% & 60% respectively
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Bovine adrenal cortex, 8200xg (51)	10mM (X3)	ACTH	8200xg and 105,000xg pellet and greatest specific activity
Beef thyroid (2)	10mM (X1.25)	Epin. prostaglandin ACTH	Chlorpromazine ($3x10^{-4}$) inhibits Epin. thyro. PGE_1 stimulation. Basal levels uneffected, F^- stimulation inhanced
Bovine & canine thyroid homogenate (52)	10mM	TSH, ACTH	TSH stimulates above F^- stimulated levels
Liver tumor (53)	8mM (X20)	Epin. & Glucagon	F^- stimulation depends on how cells grown

TABLE 1 (cont.)

Source	F^- Stimulation	Other Stimulants	Comments
Variety of dog tissue (21)	10mM		One of the original papers, many tissues assayed for AC activity
Rat fat cell membranes (61)	10mM (X3)	Epin.	GTP, GDP, DMP, CTP and UTP inhibit adenyl cyclase
Rat fat cell ghost (62)	10mM (X7)		Studied kinetics of cyclase, F^- did not affect Km, F^- increased Vmax (x10)
Renal cortex (28)	8mM	Several alkali metal cations	Agarose gel used to separate phosphodiesterase
Variety of bacteria (19)	10mM	Several nucleotides	Addition of NaF without effect
Cardiac tissue (32)	8mM (X4)	Ca^{++}, Mg^{++}, Mn^{++}	F^- increases Vmax without changing Km
Streptococcus salivarius (33)	20mM (X5)	Sulfhydryl inhibitors	Three protein peaks isolated with cyclase activity
Rat and Mouse Liver (29)	10mM (X8)	Methyl xanthines	Effect of Ca^{++} chelation studied
Variety of tissue (34)	10mM	Various hormones	Good review of early work by Sutherland

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THE DIFFERENTIAL DIAGNOSIS OF FLUORIDE DAMAGE TO PLANTS

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SUMMARY: Several typical criteria of symptoms caused by emission of damaging pollutants upon plants are demonstrated. Not all symptoms point unequivocably upon the effect of a particular pollutant. For the diagnosis of damage by air pollutants upon plants the most important criterion is the chemical analysis of leaves in conjunction with such methods as the examination by ultraviolet light of the quartz lamps and the analysis of air and rain water.

Dörries (1) and Massey (2) have pointed out the difficulties in differentiating between the symptoms due to a disease of a plant and those caused by air pollution. The author in his book Luftverunreinigung und ihre Wirkungen, 1967 (3), has established that analysis of leaves is the most reliable means of the diagnosis of air pollution damage to plants.

Whereas the current paper will compare the typical symptoms of fluoride damage with those caused by other pollutant agents, it should be emphasized that this presentation should not minimize the value of chemical analyses of the tissues of plants for the respective agents.

1. Fluorides

Damage to vegetation by fluorides has been described by numerous authors. The principal characteristics are the typical margin and tip necroses of leaves with their dark gray-brownish color and the "rolling" of the surface of the leaf. Observation of the cross-section of the leaf reveals that nearly all layers of the affected areas are destroyed (Fig. 1). Guderian, et al. (4) have carried out extensive investigations concerning damage by fluoride and induction of various symptoms in plants. They described the features of fluoride damage in a series of different plants in which the concentrations and the duration of exposure were varied.

The incipient symptoms of acute HF damage are a gray-green discoloration of the leaves which depending upon the species of the plant, turns into a necrosis with an ivory to white color, then brown to red-brown and even to brown-black. These authors demonstrated that the chronological course of an acute HF injury is determined by the dose and concentration of the damaging agent by atmospheric and other environmental conditions and by the stage of development of

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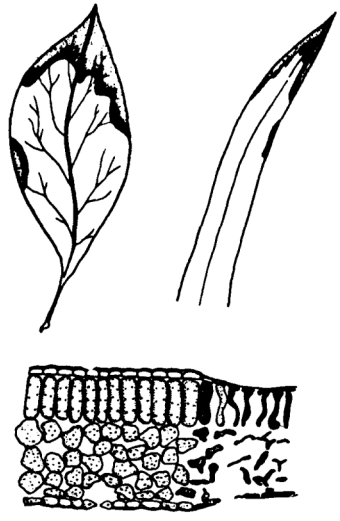
Fig. 1

Fluorine Injury



Tip and margin necrosis on the dicotyledon leaf and the monocotyledon (grass type) leaf; sharp line of demarcation; severe collapse and shrinking of the internal structure of leaf.

the plant. The leaves of blossoms are generally much more resistant but when exposed to high concentrations, may undergo bleaching. Low concentrations of HF precipitate a chlorotic bleaching of the surface of the leaf especially among legumes.

Certain species of grains and grasses exhibit marked necrosis at the tips of leaves which run along the margins. Bulb plants such as crocus, tulips, narcissus, and gladiolus also manifest tip necrosis in varying shades.

In evergreens, acute damage manifests itself in a reddish-brown necrosis of the tips of the needles. A chlorotic discoloration of the tips and of the surfaces of the leaves constitutes the beginning sign of a chronic effect due to low concentrations of fluoride. Such lesions may turn into necrosis.

Although fluorides occur in the soil at varying magnitudes - up to 30 mg per 100 grain of dry substance - ordinarily the natural fluoride content of plants is low. It ranges nearly always between 0.5 to 2 mg/100 g of dry substance, rarely to 3 mg/100 g (5 , 6). However a plant may take up from the air an amount of fluoride which causes its level to rise many times above the usual

fluoride concentrations readily determined by the qualitative analysis according to Bredemann and Radeloff (7) and by the quantitative analysis by Oelschläger (8) and Buch (9). Thus the effect of fluoride-containing emissions can be accurately determined.

Keller (10) utilizes as a criterion of fluoride damage the effect of fluoride upon peroxidase activity. Many other actions of fluoride upon enzymes have been investigated by various authors.

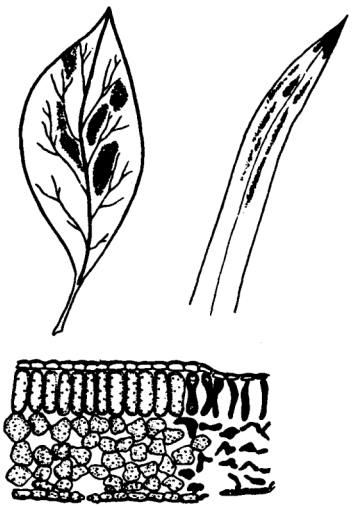
2. Sulfur Dioxide

Damage of plants by sulfur dioxide has been thoroughly investigated (11). Beginning signs of acute SO_2 damage to leaves are diffusely-green or slightly brownish colored stains which subsequently turn into brown and occasionally into black necrotic lesions. Among the dicotyledons the basic forms are the intercostal necroses. In case of low grade pollution by SO_2 small punctate necrotic areas predominate.

Among leafy trees intercostal necroses occur which sometimes take on the pattern of fish bones. The essential microscopic features of SO_2 damage

Fig. 2

Sulfur Dioxide Injury



Blotchy interveinal areas on dicotyledon leaf;
streaked areas on monocotyledon leaf (grass type).

are revealed through the cross-section namely transitional zones between the diseases and the healthy tissue (12). Such lesions do not occur when leaves dry up naturally or are exposed to frost. The palisade cells may assume the shape of dumbbells (13).

Among the monocotyledons the tip necrosis is the main characteristic of SO_2 lesions. The transitional zone from the necrotic to the green portion of the leaf shows a less conspicuous change in color than among the dicotyledons. In the conifer acute SO_2 damage manifests itself in a red-brown to fox-red discoloration of the needles which originates at the tip and also affects a limited number of localized sites near the tips.

The documentation of SO_2 damage by means of clinical analysis of leaves is not as reliable as in the case of fluoride analysis because sulfur is a part of the metabolism of the plant and because the natural sulfur content of a plant varies widely with the stage of its development. Therefore it is necessary to determine the total sulfur content of the plants and to carry out analyses of air and rainwater (14,15). For qualitative analysis for sulfur in plants I wish to refer to Bredemann and Radeloff (16). For quantitative assays for sulfur various methods are available (14). Dassler and Ewert (17) determine the SO_2 content of portions of plants by means of polarography. Biochemical assays do not appear to be sufficiently specific.

3. Hydrochloric Acid and Chlorine

Lesions on leaves caused by HCl and Cl are similar to some extent to those of fluoride damage. However they can be distinguished because of their brighter color. Microscopic examinations by Tiegs (18) demonstrated that tissues of the intercostal zones of leaves shrink considerably following exposure to chlorine. Liegel and Oelschläger (19) determined experimentally the effect of chlorine upon spinach and lettuce. Acute chloride emissions elicit at first typical chlorosis. Damage can be documented by the high chlorine content of the affected plant material according to the methods of Von Weihe (20) and Garber (21). Through chlorine-containing salt such as sodium chloride (NaCl) similar damge may occur (17).

4. Nitrose Gases and Ammonia

Nitrose gases and ammonia induce discolorations similar to those caused by SO_2; the lesions also simulate HCl damage, but are darker in shade due to the presence of tanning substances. The discoloration originates usually at the edges of the leaf, but also occurs occasionally at the intercostal spaces. In conifers the needle tips turn brownish-red (22, 23). A qualitative assay method for NH_3 was described by Bredemann and Radeloff (24) and a quantitative assay by Engel (25).

In experiments on how NO_2 affects plants Van Haut and Stratmann (26) observed gray-green or slightly brown colored spots on the leaves which they considered incipient symptoms. In broad-leaf plants the intercostal necroses pre-

dominate. The monocotyledons exhibit mainly yellow-white necrosis which originate at the tip of the leaves. Evergreens manifest red-brown to fox-red colored lesions at the tips of the needles. Kandler and Ullrich (27) presented an assay method for the differentiation of NO_2 from SO_2.

5. Fumes of Tar

Tar fumes precipitate typical changes in plants, namely a glossy lacquer-like surface of the leaves and a black to brownish discoloration which is due to the presence of tanning substances. The analytic method by Dvorak (28) consists in extraction with benzol and in ultraviolet light resulting in a blueviolet and yellowgreen fluoresence. According to Halbwachs (29) the intensity of the light of ultraviolet rays determines the degree of damage.

6. Ozone

Ozone is the most important constituent of the Los Angeles smog. By itself it causes evenly distributed white or brown patches or dots on the sur-

Fig. 3

Ozone Injury

Spotting and stippling lesions on leaf. On sectioning only the palisade layer is affected.

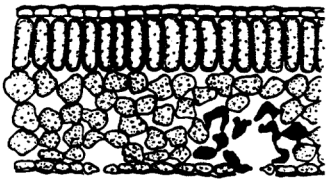
faces of the leaf. Such spots are prevalent in tobacco plants particularly in the species "Bel W 3" which serves as a test plant (4).

7. Smog

Smog damage is precipitated by a mixture of oxides of nitrogen, SO_2, ozone, hydrocarbons and carbon monoxide, which form peroxy acetyl-nitrate (PAN) under the influence of sun rays. In dicolyledons smog induces silver-white to bronze-colored changes of the surface. Leaves of grasses (Poa annua) and grains show horizontal stripes. Beans, Petunias and especially Poa annua

Fig. 4

Smog-Type Injury



Localization of lesion determined by age of leaf. On sectioning, initial collapse occurs in region of stomata.

serve as test plants. In distinction to ozone which involves mainly the palisade tissue, smog affects the base of old leaves and in young foliage the tips of leaves (13). Nielsen, Benedict and Holloman (30) described a specific test for damage by smog namely a fluoresence of the damaged leaf surfaces in ultraviolet light. Bobrov (31) utilized the colorimetric test, namely a change in color of damaged cells by thionin in order to distinguish it from damage due to other sources such as SO_2, frost and parasites.

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Discussion

Dr. Waldbott: What agents can damage vegetation other than those which you have named?

Dr. Garber: Molybdenum and selenium are known to damage plants but,to date, little is known about the characteristic features of such damage.

Dr. Cecilioni: How can one determine the damage to vegetation by a particular pollutant, when confronted with a combination of pollutants such as F, SO_2, O_3, CO_2, etc. Does one rely upon the appearance of the leaves or upon analysis?

Dr. Garber: Analysis of the leaves and rainwater may give the answer. Fluoride is usually the pollutant responsible for principal damage to vegetation.

Dr. Scholl: According to observations which I made during the past summer, damage to vegetation along roads was due to exhaust fumes, rather than to fluoride. The discoloration of the leaves and early leaf drop, especially after a dry summer,resembles fluoride injury.

Dr. Garber: Again, I should like to say that fluoride compounds are not solely responsible for damage - fluoride emitted from nearby factories can be combined with lead compounds, hydrocarbons from exhaust fumes and salt used on roads during the winter.

Dr. Oelschlager: I agree with Dr. Garber, that some of the damage to vegetation is caused by exhaust gases combined with hydrocarbons, lead, etc. rather than to fluoride alone, the concentration of which is often low. Nitrous oxides as well as CO, SO_2 and O_3 are also discharged into the air CO does not oxidize readily into CO_2.

TOXICITY OF THE METABOLITES OF FLUORINATED ANESTHETICS

by

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SUMMARY: Studies of the metabolism of fluorinated anesthetics have revealed more toxic agents than the parent compounds. Both in the case of halothane and methoxyflurane the metabolic products induce tissue damage, although with low frequency, and probably each with quite different mechanisms. Because the metabolism of these two widely used inhalation anesthetics can occur instantaneously, repeated anesthetics at short intervals may be dangerous. Therefore, the development of fluorinated anesthetics which are not metabolized in the body should be a progressive step in the direction of anesthetic safety.

Metabolism

Fluroxene (trifluoroethyl vinyl ether), tested on human volunteers in 1953 (1) and introduced into clinical practice a few years later (2), is the first fluorinated anesthetic of importance. Halothane (2-bromo-2-chloro-1, 1, 1-trifluoroethane) (3) and methoxyflurane (2, 2-dichloro-1, 1-difluoroethyl methyl ether) (4) soon followed as the result of extensive screening among the fluorinated hydrocarbons and ethers for use as anesthetics. Recently two new fluorinated ethers, enflurane (Ethrane, 2-chloro-1, 1, 2-trifluoroethyl difluoromethyl ether) and Forane (1-chloro-2, 2, 2-trifluoroethyl difluoromethyl ether) have undergone extensive clinical trials. Enflurane will probably be available for general use in 1973. Not until 1964 was evidence presented that halothane and methoxyfluorane are metabolized (5), and in 1967 it was reported that fluroxene is metabolized in animals (6).

Halidases can hydrolyze the C-Cl bond and probably also the C-Br bond (7). The C-F bond has not, however, been found to undergo enzymatic cleavage in man, although some evidence has been produced of the occurrence of such enzymes in bacteria (8). In the metabolism of methoxyflurane, the C-F bond is secondarily ruptured, but probably not through enzymatic activity. The C-F bond becomes weaker in the presence of an oxygen atom bound to the same carbon (7). Indeed, methoxyflurane seems to be the first compound in which rupture of the C-F bond has been shown to occur with certainty in man.

In ethers, the oxygen linkage is enzymatically ruptured (5) and is often the primary site of attack.

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Fluroxene

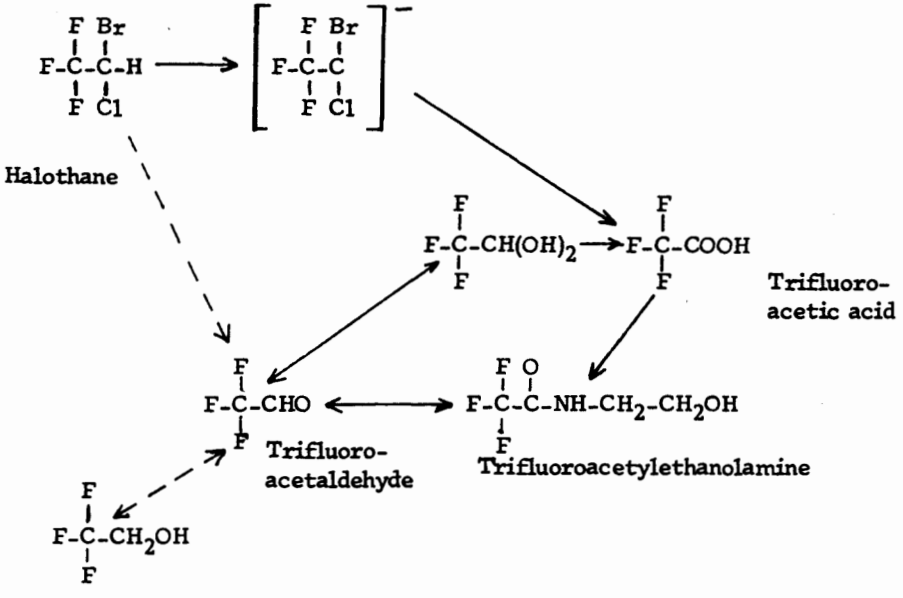
Blake and co-workers (6) found that fluroxene was transformed to CO_2, trifluoroethanol, trifluoroacetic acid and some minor unidentified metabolic products in the mouse and the dog. The CO_2 was derived from vinyl carbons only. Trifluoroethanol was considered to be an initial metabolite and accounted for 20 to 50 % of the injected fluroxene in 48 hours. Ten to 20% of the dose was recovered as trifluoroacetic acid. In man, 12.1% and 15.4% of the nonvolatile radioactivity were found in urine during 24 hours after injection of ^{14}C-fluroxene (9) and the relative amounts of trifluoroethanol and trifluoroacetic acid varied greatly in these two volunteers.

Halothane

The metabolism of halothane, requiring NADPH and O_2 (10), proceeds via debromination (11) and dechlorination to trifluoroacetic acid (12), which appears in urine (13). Trifluoroethanol and trifluoroacetaldehyde have been suggested as intermediate metabolites (14), but have not been identified. When measured in urine as trifluoroacetic acid about 12% and, as bromide 17 to 20% of the halothane is metabolized in man (15). Other metabolic pathways have also been suggested, leading to trifluoroacetic acid through an unstable ionized compound (16) or through trifluoroacetaldehyde (17,18) (Fig. 1).

Fig. 1

Probable Metabolic Pathways of Halothane



Recently Cohen (19) has identified trifluoroacetylethanolamine in the urine of a patient into whom he injected ^{14}C-halothane. The ethanolamine probably stems from the phosphatides in cell membranes (20).

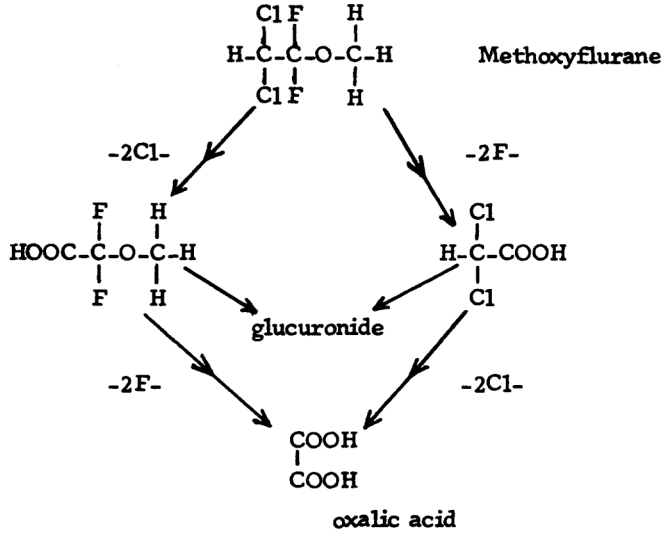
Methoxyflurane

Van Dyke and co-workers (5) showed that 3 to 6% of the injected ^{14}C-methoxyflurane, labeled in the methyl group, was converted to $^{14}CO_2$ in the rat. Inorganic chloride recovered from urine as ^{36}Cl approximated 3% of the injected dose of ^{36}Cl-methoxyflurane. Eighty-five to 90% was exhaled unaltered in 30 hours.

Holaday and co-workers (7) studied the metabolism of methyoxyflurane in two humans. Only 29% and 35% respectively of the absorbed methoxyflurane were exhaled unaltered by the two subjects. Metabolic products, indentified after exposure to methoxyflurane labeled with ^{14}C in the methyl position, were CO_2, fluoride ion, dichloroacetic acid and methoxydifluoroacetic acid. Seven to 21% underwent cleavage of the ether linkage and, in one subject, about 40% was dechlorinated and oxidized to methoxydifluoroacetic acid. Mean 24 hour urinary inorganic fluoride excretion has been found to be markedly increased in human patients anesthetized with methoxyflurane, from 67 umol/day preoperatively to 4760 µmol on the first day following anesthesia (21). Oxalate, the end-metabolic product (Fig. 2), has been identified earlier within renal tubules

Fig. 2

Probable Metabolic Pathways of Methoxyflurane



at autopsy (22, 23) and recently Mazze and co-workers (21) found increased urinary excretion of oxalate in patients anesthetized with methoxyflurane.

Enflurane and Forane

Enflurane is metabolized less than methoxyflurane and halothane in experimental animals (24, 25), and in man only 2.4 % of the administered enflurane was recovered as urinary fluorine (7). Pig liver-perfusion studies have not revealed any metabolism of forane (25). To date no results from studies on man have been published.

Toxicity

The acute toxicity of the fluorinated anesthetics seems to be due to their anesthetic potency, whereas a major part of their delayed and chronic toxicity may be caused by the metabolites.

Fluroxene

Trifluoroethanol, the primary metabolite of fluroxene, showed a considerable delayed toxicity (195 mg/kg intraperitoneally) in mice according to Airaksinen and co-workers (18). Trifluoroacetate was almost nontoxic and the supposed intermediate metabolite, trifluoroacetaldehyde hydrate, was intermediately toxic (18, 26, 27). The toxicity of these metabolites seemed to be due to formation of trifluoroacetaldehyde, or its close derivatives, which block SH-enzymes and inhibit energy production (18, 26, 28, 29).

Dose-dependent fat accumulation in the livers of mice occurred after single administration of trifluoroethanol and trifluoroacetaldehyde hydrate, but no necrosis of the liver cells was seen even after treatment for two weeks (28). Morris reported no liver injury in man after fluroxene anesthesia (30), and histological studies have not revealed any liver damage in dogs 60 minutes after anesthesia with fluroxene (1). Quite recently, however, a few cases of liver necrosis following fluroxene anesthesia have been observed in patients receiving antiepileptic treatment (drug interaction?) (31).

Trifluoroethanol, trifluoroacetaldehyde hydrate and trifluoroacetic acid did not cause any histological changes in the heart and kidneys as judged by light microscopy (28).

Halothane

Trifluoroacetic acid was almost nontoxic when administered as sodium salt (26). As a strong acid, trifluoroacetic acid is almost completely ionized at physiological pH (27) and therefore does not penetrate biological membranes. Its formation in vivo inside the cells may, however, damage tissues. Trifluoroethanol and trifluoroacetaldehyde hydrate, two suggested metabolites of halothane (14), were discussed under fluoroxene and were considered hepatotoxic in

mice (28). Trifluoroacetic acid also caused a moderate accumulation of fat in the livers, thus explaining the increase in liver weight found by Schimassek and co-workers (32).

Unidentified metabolic products of halothane accumulated increasingly in the livers of mice after repeated exposures to the anesthetic (33), and therefore the concentration of some of the metabolites may rise to a toxic level. Trifluoroacetyl and trifluoromethyl groups have been identified in mass spectrograms of the blood of rats exposed to or injected with halothane (34). The metabolites in the body are probably bound to different $-NH_2$ groups (18,19) and also to - SH groups (18,28) in peptides, proteins including enzymes, and cell membrane components.

Methoxyflurane

Both renal and hepatic injuries (separately or simultaneously) have been reported after methoxyflurane anesthesia (35,36,37,38,39). Fluoride and oxalic acid are nephrotoxins (40), and poisoning with a mixture of oxalic acid and hydrofluoric acid has caused hepatic damage (41). In a few cases tetracycline seems to be associated with methoxyflurane in causing renal damage (42).

Serum concentrations of fluoride above 200 µmol/l have been described to be nephrotoxic (40) and peak values of 270-275 µmol/l (21, 40) have been measured after methoxyflurane anesthesia in patients developing nonfatal kidney failure. On the other hand, in fatal sodium fluoride intoxications the serum fluoride concentration has sometimes been as low as 160 µmol/l (43).

Oxalate crystals are often seen in renal biopsies from kidneys affected by methoxyflurane (35). Very little is known about the toxicity of the other metabolites of methoxyflurane. As free acids both dichloroacetic acid and glyoxalic acid, as well as the end metabolite, oxalic acid, are irritants to mucous membranes (44), but very little evidence is available about their toxicity to internal organs. The LD_{50} of dichloroacetic acid equals those of acetic acid and trichloroacetic acid (45). Glyoxalic acid is rapidly converted to oxalic acid, which is also found in blood and urine in ethylene glycolic poisonings (46).

The Role of Metabolites in the Toxic Effects of the Anesthetics

Only rarely have cases of hepatic damage following fluroxene anesthesia been reported (31). Fluroxene has not, however, been as extensively studied or as widely used as for example halothane, although its anesthetic potency and circulatory effects seem to be favorable.

The rare occurrence of liver injury in man after halothane anesthesia is probably due to its metabolites (33, 34). Formation of reactive acetyl and acetate groups in metabolism allows binding to cell membrane components (19) and hapten immunization (47) of the metabolites. The positive lymphocyte stimula-

tion test (48) and demonstration of antimitochondrial antibodies in the serum of some of the patients with icterus after halothane anesthesia do not necessarily prove that the liver injury is on an allergic basis but suggest at least a concomitant immunological reaction (49). Because most of the patients with liver injury after halothane anesthesia have been exposed at least twice to the anesthetic, avoidance of halothane has been recommended in emergency cases and of its second time use within 1 to 2 months after the first anesthesia (50).

Oxalate crystals in the urine and precipitation in the renal tubules do not appear to be pre-requisites for the diagnosis of renal injury caused by methoxyflurane. The polyuric syndrome (51,52) is either related to unresponsiveness of the kidneys to ADH (vasopressin) or to failure of the renal medulla to maintain a hypertonic state; it may be caused by the liberated fluoride (51). On the other hand, both fluoride and oxalate are enzyme inhibitors, depressing energy metabolism and active ion transport in the kidneys (20,53). The liver injury sometimes seen after methoxyflurane anesthesia is even more obscure. In rare cases, cross sensitization of the liver between halothane and methoxyflurane has been suspected, but the relationship is unclear (54).

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The policy of this journal is to refrain from entering the controversies over fluoridated water and air pollution. However, Drs. B. Houwink and G. W. Kwant of Utrecht, Netherlands, requested time to present their data on the fluoridation experiment in Tiel at the Fourth Conference of the International Society for Fluoride Research at The Hague, October 1971. Dr. Kwant submitted his manuscript for publication in FLUORIDE. It is herewith presented, accompanied by a critique by Dr. Rudolph Ziegelbecker, Graz, Austria and Mr. Howard Thomson, North Andover, Massachusetts.

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SIXTEEN YEARS OF WATERFLUORIDATION IN THE NETHERLANDS AND ITS INFLUENCE ON DENTAL DECAY

by

G. W. Kwant
Utrecht, Holland

SUMMARY: For the Dutch fluoridation project, the towns of Culemborg and Tiel were selected after sociological screening. Tiel was fluoridated up to 1.1 mg per liter in March 1953, and compared to Culemborg with a fluoride concentration of 0.1 ppm in its drinking water. In this experiment, caries was diagnosed in different sites. This method permits a better insight into the total number of cavities in a dentition. It also shows that fluoridation does not have the same effect on all tooth surfaces. Caries of the proximal surfaces was diagnosed with the aid of X-rays, whereas all other sites were evaluated clinically. The main study groups, consisting of children 11 to 15 years of age, were examined every second year. Each age class in these groups consisted of about 70 boys and 70 girls. All these children were born and had resided either in Tiel or in Culemborg all their lives. During the course of these sixteen years, the caries frequency in Culemborg has increased whereas it has decreased in Tiel.

This study on the influence on dental decay of artificial fluoridation of domestic water supplies started in 1952. Of the two towns to be compared, in Tiel the drinking water has been fluoridated since March 1953 at 1.1 mg F/l, whereas Culemborg served as comparison town (0.1 mg F/l).

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Presented at the Fourth Annual Conference of I. S. F. R., The Hague, 10/24-27/71.

The data were collected according to the clinical trial method in which three types of lesions were differentiated: lesions in pits and fissures (mainly occlusal surfaces), lesions in proximal surfaces (the surface between adjoining teeth) and lesions of the free smooth buccal and lingual surfaces (mostly along the gingival margin). Data for the different sites are presented separately. This is not done only for anatomical or clinical reasons. Barr et al. stated in 1957 that the intrapatient correlation of these types of caries is generally poor. Moreover, it is known that lesions in these different sites are not influenced to an equal extent by fluorides.

Caries of the proximal surfaces of the posterior teeth and the upper front teeth was diagnosed exclusively from radiographs. These X-rays were made according to a standardized technique. This method has proved to be more accurate for these surfaces than the intra-oral examination method. Also one great advantage of the method employed was that it made a blind evaluation possible: the radiographs of Tiel and Culemborg were mixed and examined at random.

For pit and fissure caries and for the free smooth surfaces, a standardized clinical method was used. These surfaces were evaluated in duplicate by two dentists independently. The clinical examinations took place alternately in Culemborg and Tiel to avoid a shift in standards between the two.

The data in this paper concern the dentine (d)-lesions which should be filled or have been filled. A special difficulty in the evaluation was raised by the extracted teeth. Until the 1959 survey, the number of extractions was about the same in Tiel and Culemborg. But in the later years of the experiment a discrepancy between the number of extractions in Culemborg and Tiel arose. Extractions per child in children born in 1953 and 1954 in Culemborg were 1.8 and 1.7 whereas in Tiel they were 0.4 and 0.5 respectively. In other words, in Tiel extractions were more than 70% less than those in Culemborg. In most cases this difficulty was settled by introducing into the data the diagnosis that was presented in the survey in which the extracted tooth was present for the last time.

Results

For proximal surface lesions, every other year the teeth of 11 to 15 year-old children were examined. In 1968 children of these age groups had 5.6 proximal lesions per child in Culemborg, 1.4 in Tiel. In 1953 when fluoridation started the corresponding figures were 4.0 and 4.1.

Figure 1 gives the number of lesions per child for Culemborg and Tiel respectively for each age group in this classification and the percentage reduction in cavities in Tiel. The dots indicate the situation in 1952 before fluoridation started when there were no significant differences between Tiel and Culemborg. The minor differences appear to stem from the random composition of the groups. During these 16 years, the number of lesions in the nonfluoridated control group have risen whereas in Tiel the numbers declined.

Between 1952 and 1969, twenty-eight groups of children 15 years of age have been examined. Figure 2 gives the number of proximal d-lesions per child. The upper part of the curve shows the percentage reduction in d-lesions in Tiel. The last three groups show about 70% less d-lesions for Tiel. The number of cavities in Tiel seems to be constant, which implies that the children born in 1952 have already obtained a maximum reduction in lesions. This figure shows the increase in lesions in Culemborg as well as the decrease in Tiel during sixteen years. Also the first measurable differences are found about four to five years after the start of fluoridation.

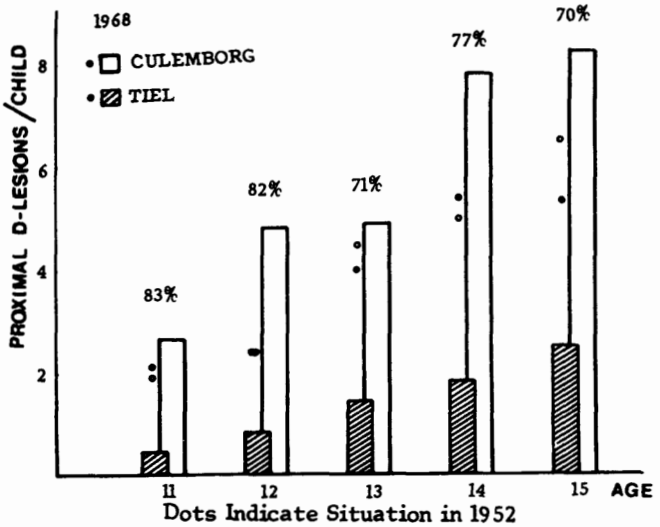
Figure 3, using data derived from a longitudinal study, shows the difference in caries increment between children born in 1954 in the fluoridated town and in the control town. In each group exactly the same children were present during all these years. For the Culemborg children, the mean increase is 1.2 lesions per year on this site, for Tiel 0.3 lesions.

Figure 4 presents (in the upper part) the effect on pit and fissure lesions of molars and premolars. The clinical inspection was done in 10 groups of children. These data have also been derived from longitudinally followed groups.

The effect is less impressive than in the proximal surfaces. The Tiel children born in 1953 and 1954 have 36% and 38% less lesions respectively. From the current data it seems that, to achieve a maximum effect, fluoride should be consumed during the early formative period of the teeth.

Fig. 1

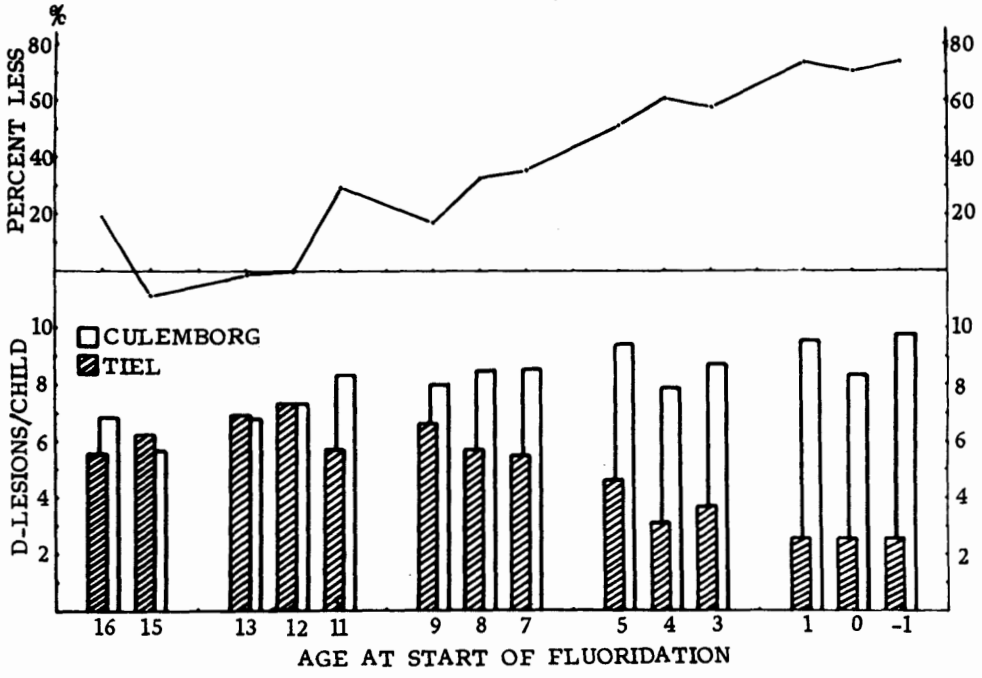
Number of Proximal D-Lesions Per Child; Survey 1968
(Percentage Reduction in D-Lesions in Tiel Compared With Culemborg)



The lower part of figure 4 shows that free smooth surfaces are protected best by fluorides (about 80 - 90% less cavities in Tiel than in Culemborg). Although the total number of cavities in this site is not very high (even in the control town, in 1968, the number of d-lesions per child of 15 years of age was 4.0), each of these lesions endangers a tooth more seriously than most other lesions. The topical effect of fluorides on these surfaces seems to be much more favorable here than on other surfaces.

Fig. 2

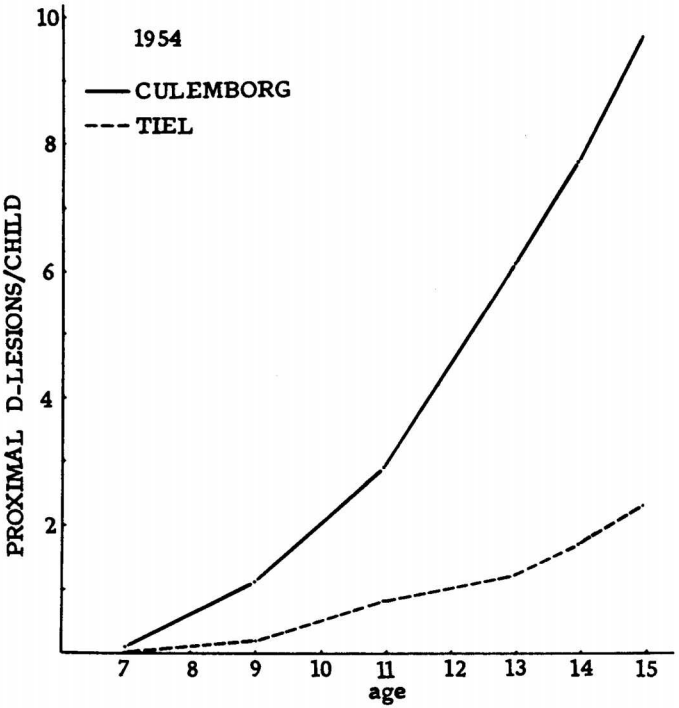
Mean Number of Proximal D-Lesions Per Child in 15 Year-Old Children
(Percentage Reductions in D-Lesions in Tiel Compared With Culemborg)



Age at start of fluoridation	16	15	13	12	11	9	8	7	5	4	3	1	0	-1
Year of survey	'52	'53	'55	'56	'57	'59	'60	'61	'63	'64	'65	'67	'68	'69
Culemborg	6.9	5.7	6.8	7.3	8.3	8.0	8.5	8.5	9.4	7.8	8.6	9.5	8.3	9.8
Tiel	5.6	6.2	6.9	7.3	5.7	6.6	5.7	5.4	4.6	3.0	3.6	2.5	2.5	2.5
% less cavities in Tiel	19	-9	-1	0	31	18	33	36	51	62	58	74	70	74

Fig. 3

Mean Number of Proximal D-Lesions Per Child
in Children Born in 1954



Age	7	9	11	13	14	15
Culemborg	0.1	1.1	2.9	6.1	7.7	9.7
Tiel	0.0	0.2	0.8	1.2	1.7	2.3

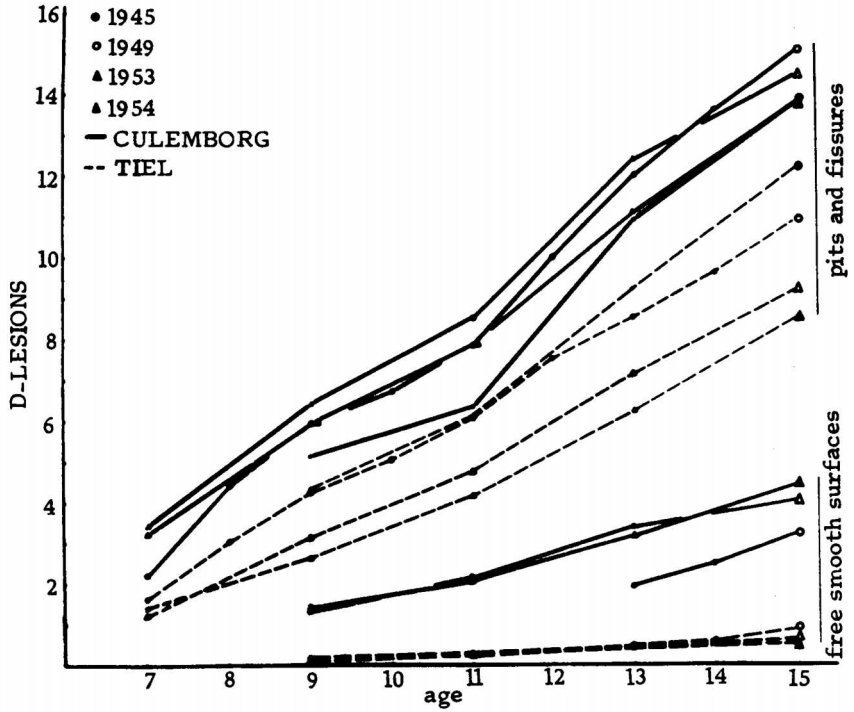
To conclude,figure 5 shows the frequency distribution of the children in Tiel and Culemborg born in 1954 over the caries scores. In this figure the extractions have not been calculated. It is clear that this distribution favors the fluoridated town. From the cumulative curve,one can see that in Tiel 50% of the children have less than 12 cavities, compared to 12% in Culemborg. In Culemborg 50% of the children have more than 23 cavities, compared to less than 10% in Tiel.

At the age of 15, there were more than 60% less cavities in Tiel than in Culemborg.

Because of the time limit only a minor number of the data found could be presented. Nevertheless it is obvious that from the dental point of view, fluoridation has a beneficial effect on human dentition.

Fig. 4

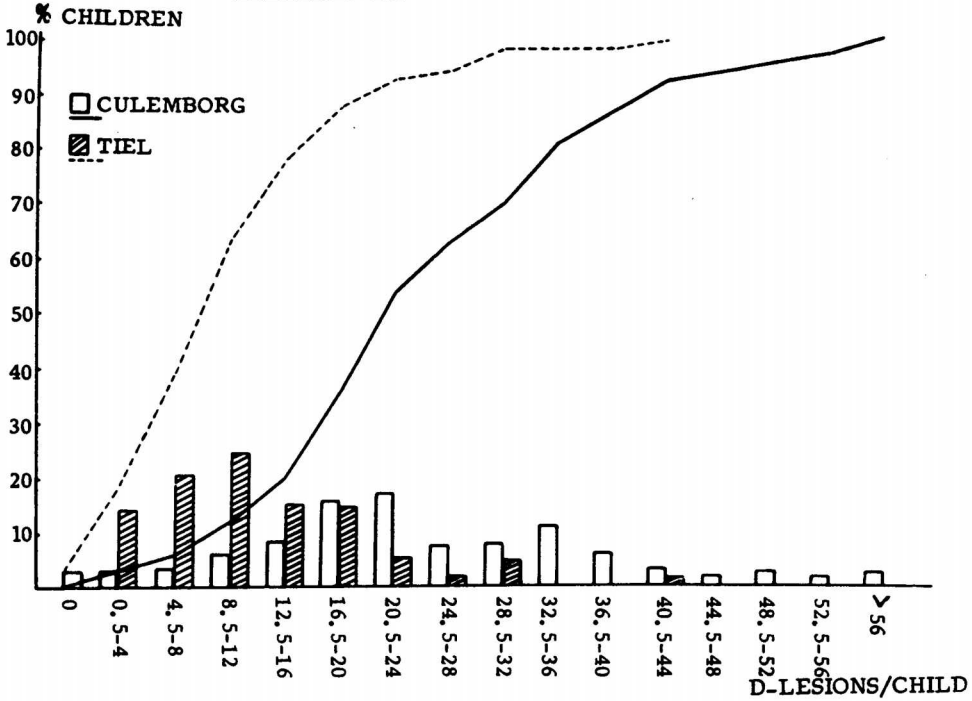
Mean Number of D-Lesions Per Child in Pits and Fissures and in Free Smooth Surfaces in Different Age Groups



	Year of birth	1945 C	1945 T	1949 C	1949 T	1953 C	1953 T	1954 C	1954 T
	age 7			2.2	1.6	3.4	1.2	3.2	1.4
	8			4.4	3.0				
	9	5.1	4.3	5.9	4.2	6.4	3.1	5.9	2.6
Pit and	10			6.7	5.0				
fissure	11	6.8	6.1	7.8	6.0	8.5	4.7	7.8	4.1
cavities	12			10.0	7.5				
	13	10.9	9.2	12.0	8.5	12.4	7.1	11.1	6.2
	14			13.6	9.6				
	15	13.8	12.2	15.1	10.9	14.5	9.2	13.8	8.5
	age 9					1.3	0.1	1.4	0.2
	10								
Gingival	11					2.1	0.2	2.0	0.3
cavities	12								
	13			1.9	0.5	3.4	0.4	3.2	0.5
	14			2.5	0.6				
	15			3.2	0.9	4.0	0.7	4.4	0.5

Fig. 5

Distribution of D-Lesions and Corresponding Cumulative Curves in Children Born in 1954



Number of D-Lesions	% of children		cumulative curve	
	Cul.	Tiel	Cul.	Tiel
0	0	2.7	0	2.7
0.5 - 4.0	2.9	15.7	2.9	18.4
4.5 - 8.0	3.0	20.4	5.9	38.8
8.5 - 12.0	5.9	24.5	11.8	63.3
12.5 - 16.0	8.1	14.9	19.9	78.2
16.5 - 20.0	15.6	9.5	35.5	87.7
20.5 - 24.0	18.6	4.8	54.1	92.5
24.5 - 28.0	8.7	1.4	62.8	93.9
28.5 - 32.0	7.4	4.7	70.2	98.6
32.5 - 36.0	11.1	0	81.3	98.6
36.5 - 40.0	5.8	0	87.1	98.6
40.5 - 44.0	5.3	1.4	92.4	100.0
44.5 - 48.0	1.5	0	93.9	
48.5 - 52.0	2.3	0	96.2	
52.5 - 56.0	1.5	0	97.7	
>56.0	2.3	0	100.0	

Discussion

Dr. Oelschläger: You have discussed the question of fluoridation of water for children. Have you considered that adults may take in up to 40 mgm of fluoride daily, especially industrial workers and those who reside near fluoride-emitting industries? Very high fluoride levels have been found in their garden-grown vegetables.

Dr. Kwant: I agree with you that we have to fight pollution of all types. But, we must differentiate this problem from fluoridation of water supplies in nonpolluted areas. In polluted areas, tests should be made to find out the amount of fluoride present in water and food in order to determine the proper amount of fluoride needed.

Dr. Waldbott: Were the amounts of calcium, magnesium, and other constituents in the water the same in both experimental and control towns? - Have you taken into consideration the well established fact that fluoride delays tooth eruption, that it thus also delays the appearance of tooth decay? In view of this delay any comparison of fluoridated and nonfluoridated teeth of the same age group is fallacious. Many, if not all, U. S. A. studies, designed to show a 60% reduction in tooth decay contain the same fallacy.

Dr. Kwant: Yes, the water supplies of both towns contained the same minerals and salts, because the soil is much the same. - No, there is no delay in the eruption of teeth. Even after 5 to 10 years of fluoridation, as well as in the 15 year-old children there still is a drop in dental decay up to 60%.

Dr. Mohamed: You stated that two dentists tested the children independently and X-rays of the teeth were taken separately. You cannot take a mean on such a comparative test, unless the same score is used on all the children tested. You cannot average the dentists' readings or opinions. Averages do not hold, if the readings are different.

Dr. Kwant: Both towns were calibrated against the same degree of caries. We used X-ray independently, because this is better than findings by the visual eye.

Dr. Cooke: What percentage of dental fluorosis or discoloration of the teeth did you find in the fluoridated town among the 15 year-old children?

Dr. Kwant: Yes, we found some among the 15 year-old children.

Dr. Jolly: In our experience, we have seen dental fluorosis in people in areas where the fluoride content of the water was less than 1 ppm - where the water only contained 0.7 ppm fluoride. After fluoridation for 30 to 40 years you may find delayed ill-effects. Observation over fifteen or 25 years is not a long enough period of time to arrive at a decision concerning the long-term effects of fluoridation.

COMMENTS ON THE PAPER BY G. W. KWANT

SIXTEEN YEARS OF WATER FLUORIDATION IN THE NETHERLANDS AND ITS INFLUENCE ON DENTAL DECAY

by

R. Ziegelbecker* and H. M. Thomson**
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SUMMARY: The article "Sixteen Years of Water Fluoridation in the Netherlands and its Influence on Dental Decay" by G. W. Kwant which purports to prove fluoridation a success in the Netherlands exhibits serious shortcomings. They pertain to the following points: 1. selection of an adequate control city, 2. proper sampling of the children, 3. lack of essential data, and 4. faulty interpretation of the results. Factors other than fluoride were given no consideration nor have the long-term effects on older children and adults been evaluated, which are requisites for conclusions regarding the total dental effects.

In the following an analysis is presented of the statistical findings by Kwant respecting the Netherland's Tiel/Culemborg survey and of those of Grand Rapids, Michigan after 10 years of fluoridation, which provide the basis for fluoridation in Holland.

1. Selection of Adequate Control

During the years of the study, between 1950 and 1970, Culemborg's population increased by 39.6% (4372 persons) whereas that in Tiel increased by 53.6% (7612 persons). The increment in Tiel's population was therefore 75% larger than that of the control city of Culemborg. The Tiel population was 28.5% larger than that of Culemborg in 1950 and 41.4% larger in 1970 (Fig. 1). These differences in population and population growth should have been taken into account in computing the caries figures in the two cities.

Moreover, Kwant's article provides no information about the mineral composition of drinking water in each community nor about the industrial activities of either community. Contamination of food by atmospheric fluorides in an industrial area affects materially the total fluoride uptake into the human body. Therefore any comparison made solely on the basis of the fluoride content of drinking water cannot be considered valid.

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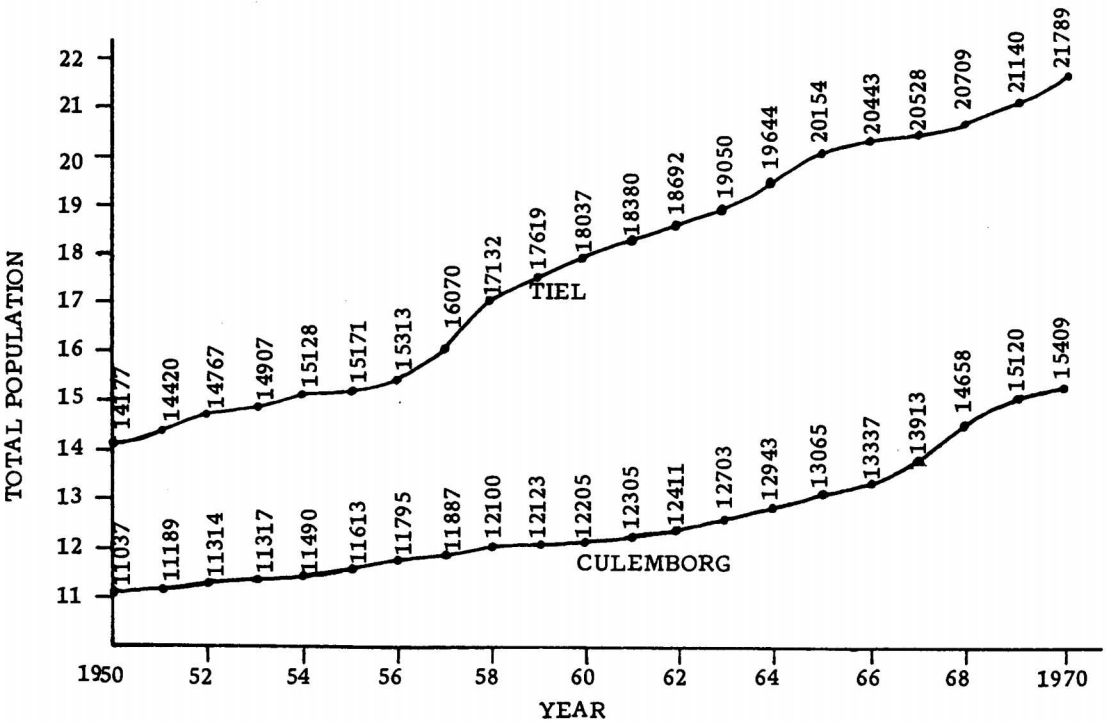
2. The Sampling of the Population

The sampling of children in fluoridated Tiel for comparison with unfluoridated Culemborg is not representative of the entire population but only of a very small portion. No information is given on how the children were selected for the study. There is no indication that the children who were examined were a representative sampling of the total child population of the two cities. Furthermore, the survey covers only about 60 to 75 boys and a similar number of girls per year. Such small groups compared with 3½ million individuals in Holland who are now drinking artificially fluoridated water (of a total population of more than 13 million) do not provide representative samplings.

Fig. 1

Population Growth in Tiel and Culemborg



Data obtained from the Central Bureau for Statistics in Holland

Therefore the findings on the limited samplings in Tiel and Culemborg cannot reasonably be extrapolated to the total child population of the Netherlands, nor even to the total child population of either city, nor to any of the adult population.

3. Incomplete Data

Kwant attributes the computed 70% reduction in caries in the 13 and 15 year-old children of fluoridated Tiel compared with children of equal ages in Culemborg, to the action of fluoride added to drinking water. The data which have been presented are not sufficient to warrant such a conclusion.

In Kwant's figure 1, data on children between the 6th and 10th year of life are missing. Data for ages well beyond 15 are required because loss of teeth from periodontal disease should be taken into account in evaluating the overall effect of fluoridation.

In Kwant's figure 2, data on 15 year-old children examined in 1954, 1958, 1962, and 1966 are missing, as shown in our Table 1. The available figures do not indicate gradual progress in the reduction of tooth decay in the Tiel children, but an erratic fluctuation. For instance, in 1960 (after 7 years of fluoridation) the number of d-lesions is practically the same as in 1952 (one year before introduction of fluoridation). Such fluctuations require further explanations. There-

TABLE 1

Lesions of Dentine in Tiel 1952-1969

Year	dentinal lesions**	Year	d-lesions	Year	d-lesions	Year	d-lesions
1952	5.6	1957	5.7	1962	?	1966	?
1953*	6.2	1958	?	1963	4.6	1967	2.5
1954	?	1959	6.6	1964	3.0	1968	2.5
1955	6.9	1960	5.7	1965	3.6	1969	2.5
1956	7.3	1961	5.4				

*Initiation of fluoridation **dentinal lesions: d-lesions

fore the observed reduction in dental caries cannot be attributed solely to the addition of fluoride in drinking water.

Other factors which influence the incidence of tooth decay should have been quantitatively determined in order to establish what portion (if any) of caries reduction was due to the effect of fluoride. For instance, the number of d (dentine)-lesions in Tiel children could have declined because of the fact that these children (but not the Culemborg children) were alerted to the importance of visiting their dentists more frequently during the later years of fluoridation

than prior to it or at the beginning of the experiment.* Thus the d-lesions could have declined because small or beginning defects might have been treated and eliminated by the dentists and their penetration into the dentine could have been prevented.

In Kwant's figure 3, data are missing on dental caries findings for the 6, 8, 10, and 12 year-old children all of whom were born in 1954. Between 1960 and 1969, in nonfluoridated Culemborg the d-lesions of the 15 year-old children increased by 1.3 (see Kwant's figure 2: 8.5 d-lesions in 1960; 9.8 d-lesions in 1969), whereas the pit and fissure cavities in the same children remained unchanged (see Kwant's figure 4: 13.8 cavities in 1960; 13.8 cavities in 1969). Moreover, in Culemborg the pit and fissure cavities of the 15 year-old children born in 1954 decreased by 1.3 compared with those of the children born in 1949 (see figure 4, children born in 1949 had 15.1 cavities; children born in 1954 had 13.8 cavities) whereas, at the same time, the gingival cavities in the same children increased by 1.2 (born in 1949: 3.2 cavities; born in 1954: 4.4 cavities). These are striking statistical discrepancies which cannot be related to a single factor such as the addition of fluoride to drinking water.

In Kwant's figure 4, likewise, important data are lacking. Among the children born in 1945, 1953 and 1954, no data are presented for the ages 8, 10, 12 and 14 years nor for the 7 year-old age group born in 1945. The curve of the yearly caries increment is therefore much less reliable than, for instance, the corresponding curve for the Grand Rapids study. The annual rate of increase of "pit and fissure cavities" was approximately the same in Tiel and in Culemborg for the children born in all four years. Because the classes of 1953 and 1954 in Tiel had equal exposure to fluoridation there should have been no differences in the condition of their teeth.

Likewise, the lesions in Tiel children born in 1953 and 1954 were about the same as in the Culemborg children who were only 2 to 4 years younger. These figures indicate a delay in the appearance of the lesions rather than an actual reduction in tooth decay, a feature which can be explained at least in part by the fact that fluoride is known to retard eruption of teeth.

There is no appreciable difference in the number of the "pit and fissure cavities" in the Tiel children born in 1945 and in those born in 1949 up to age 13, even with a 4-year difference in exposure to fluoride. A strong downward trend was observed in Culemborg from 1953 to 1954 without any fluoride.

In Kwant's figure 5, the percentage of children with less d-lesions in Tiel

*Supplementary measures such as improved diet, particularly fresh fruits in season instead of sweets and soft drinks, topical application and increased dental care have been acknowledged to have been instituted in several U. S. communities e.g. Philadelphia, Chicago, Grand Rapids, simultaneously with or shortly following initiation of fluoridation.

than in Culemborg also suggests that factors other than fluoride were involved as, for instance, better dental care, avoidance of caries-inducing foods, and differences in the mineral composition of the water supplies in the two cities. No children in Culemborg are without d-lesions, an indication that the Culemborg children have received less dental care than those in Tiel. Adequate dental care would have prevented the d-lesions.

It appears therefore that the figures which the author has presented constitute selected data from which no proof of the efficacy of fluoride, can be established.

4. The Use of Dentine-Involving Lesions as a Measure for Caries Incidence

Kwant utilizes the decay in the dentine (d-lesion) as a measure of caries incidence: The number of such lesions is largely dependent upon the amount of dental care which the child receives. The more frequently a child is seen by his dentist or is examined either in the school or in the kindergarten by the school dentists, the lower will be the incidence of dentine caries in that child. For, the lesion can be repaired in its early stage before it penetrates into the dentine.

According to the report of the Netherlands Health Department (Gezondheidsraad) 1960, page 5, fig. IX-8, marked reduction in caries of 11 year-old Tiel children had already occurred following only two years of fluoridation.

In 1953, prior to fluoridation in Tiel, 11 year-old children born in 1942 in Culemborg averaged 2.0 dentine caries at the proximal surface compared to 2.1 for Tiel. Two years later, in 1955, the same children, now 13 years-old, showed an incidence of 4.7 dentine caries of the proximal surfaces in Culemborg and 3.3 in Tiel. This constitutes a 30% reduction in dentine caries in Tiel compared with Culemborg after only two years of fluoridation. In 1957, after two more years of fluoridation, the same children now aged 15, averaged 7.7 dentine caries in Culemborg and 5.0 in Tiel, a reduction of 35%. In the first two years of fluoridation in Tiel the increment of d-lesions at the proximal tooth surfaces of the children born in 1942 was about 125% higher in Culemborg than in Tiel but in the following two years it was only about 76% higher. Tooth decay is irreversible. Such prompt reduction in decay is not attributable to addition of fluoride to drinking water. Therefore, in children born in 1942 who were 11 years old at the start of fluoridation in 1953, a reduction of tooth decay due to fluoridation could only have taken place in teeth which are subject to caries after the 11th year of life.

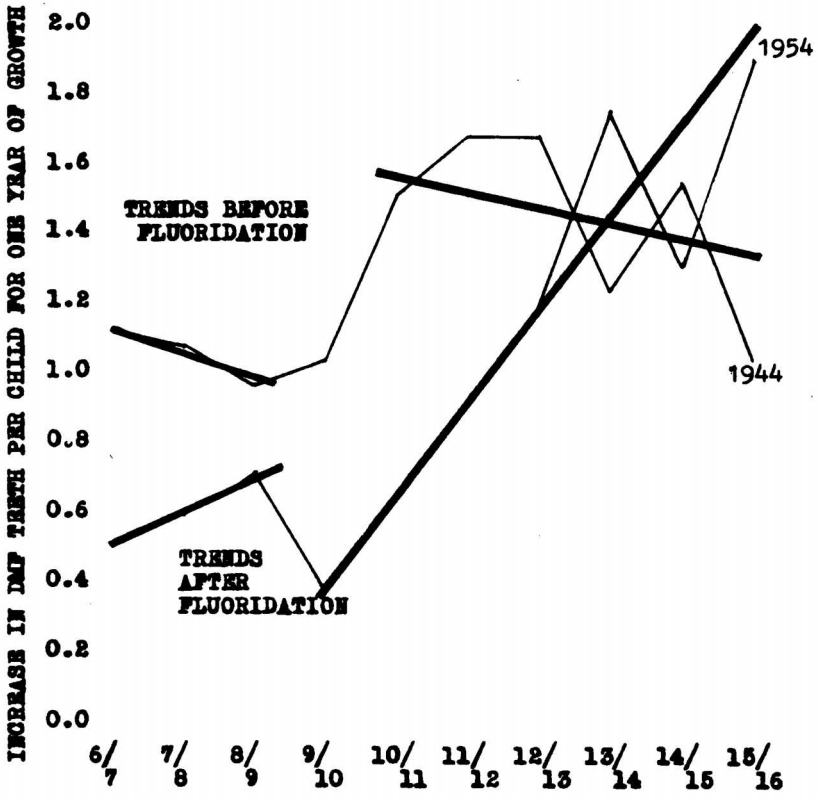
Thus the differences in dental caries between children in fluoridated Tiel and in nonfluoridated Culemborg cannot be credited to fluoridated drinking water.

5. Comparison of the Tiel Data with those of Grand Rapids

It is desirable to compare the Tiel/Culemborg findings with those of Grand Rapids because the Tiel fluoridation study is relatively small and because the Netherlands Gezondheidsraad (Public Health Service) (1) established its position on the results of the Grand Rapids, Michigan, experiment as well as upon Kwant's data. Public Health Reports (Arnold, F. A., Jr., Dean, H. T., Jay, P. and Knutson, J.

Fig. 2

Yearly Increment of Children's Tooth Decay (DMF)
In Grand Rapids, Michigan
Before and After Ten Years of Fluoridation



Increase in Decayed, Missing and Filled Teeth (DMF) Each Year

	AGE	6	7	8	9	10	11
1944	DMF	0.78	1.89	2.95	3.90	4.92	6.41
	INCREASE	1.11	1.06	0.95	1.02	1.49	1.66
1954	DMF	0.19	0.69	1.27	1.97	2.34	2.98
	INCREASE	0.50	0.58	0.70	0.37	0.64	0.89

	AGE	12	13	14	15	16
1944	DMF	8.07	9.73	10.95	12.48	13.50
	INCREASE	1.66	1.22	1.53	1.02	
1954	DMF	3.87	5.05	6.78	8.07	9.95
	INCREASE	1.18	1.73	1.29	1.88	

W., 71:655, July 1956) provides the 1944 and 1954 data for a DMF* index for permanent teeth for an average child in Grand Rapids, Michigan. Additional data for ages 12 to 16 in 1959 are given in the Journal of the American Dental Association, vol. 65, p. 782, December 1962. The Grand Rapids DMF index data are reproduced in Fig. 2. The increase for each one-year interval is shown in the same figure.

Year to year DMF differences before and after fluoridation are plotted as points in the chart, against the age intervals from ages 6-7 to ages 15-16. Thin lines connect the points.

Before fluoridation the trends were down: the annual increase tended to get smaller. After ten years of fluoridation the trends were sharply up: teeth were decaying at a faster rate. This shows again the vital need for comparable observations beyond age 16. If these trends were to be extrapolated indefinitely the teeth of adults would be expected to be worse after fluoridation than before.

A secondary conclusion can be drawn. The zig-zag shapes of DMF curves and yearly increases in DMF teeth demonstrate the statistical hazard of placing confidence in DMF figures for any one year.

The data of the yearly caries increment therefore demonstrate that fluoride in drinking water has no long-term beneficial effect on teeth and that the observed reduction of caries - as in the Tiel/Culemborg study - must be attributed in whole or in part to factors other than fluoride.

Conclusion

An analysis of the figures of the Tiel/Culemborg survey in the Netherlands and those of Grand Rapids, Michigan, in U.S.A. after 10 years of fluoridation which provides the basis for fluoridation in Holland shows that the claim of benefits to teeth from fluoride added to drinking water is not supported by the data presented.

Bibliography

1. The Netherlands Gezondheidsraad, 1960, p. 25 and 1970, p. 14.

* Decayed, missing and filled teeth

RENAL FAILURE AND FLUOROSIS

by

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(Abstracted from the Journ. Amer. Med. Assoc., 222:783-85, 1972)

At the outset of this paper the authors emphasize that fluoridation is generally agreed to be safe in persons with normal kidneys. However they considered it a reasonable possibility that systemic fluorosis might occur in individuals with diminished renal function. Their report deals with two such cases, 18 and 17 years-old respectively. The 18 year-old boy had been drinking fluoride containing water throughout his life from two sources. Most of his water came from an artesian well which contained 2.6 ppm, his city's water contained 0.4 ppm. A sample of water consumed by the second case, a 17 year-old girl, contained 1.7 ppm fluoride. In neither case report was the exact duration of the fluoride water intake nor the complete chemical composition of the drinking water given.

Case 1 had always experienced an extraordinary thirst, drinking approximately two gallons of water daily. Urinary symptoms had been unusually sparse and inconspicuous. At times albumen was found in the urine but no other indication of kidney disease was reported. The family history was negative for renal disease. The teeth were mottled and "very opaque"; several had "peg tips"; all were caries-free. There was loss of mandibular structure and possible loss of the cortex of tooth sockets.

Laboratory tests showed slight (grade 2) proteinuria and a reduction in creatinine clearance to 26 ml/min (normal 100). The para-aminohippurate clearance (C_{pah}) was 118 ml/min. (normal 600) and the inulin clearance was 26 ml/min. An excretory urogram revealed widening of the collecting system of the right ureter and an atrophic right kidney; the left kidney was not visualized. X-rays of the hips, pelvis, and lumbar spine showed increased density of the bones. A slight decrease in serum calcium (10.3 mg/100 ml) and phosphorus (5.9 mg/100ml) in the serum immunoreactive parathyroid hormone (139 ul eq/ml), as well as in the serum alkaline phosphatase (99 IU/liter) was observed. Other laboratory tests were unremarkable. The boy's water intake was reduced to 1 gallon of fluoride-free water per day but his kidney function failed to improve.

The second patient developed symptoms four months prior to her admission to the Mayo Clinic with a gradual onset of a dull aching pain in the right flank and with urinary frequency. At that time she had protein, blood, pus, and bacteria in the urine; this condition improved with penicillin treatment. Three weeks prior to admission, the pain recurred with fever, nausea, vomiting and

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dysuria. These symptoms again improved with antibiotic treatment but the blood urea nitrogen increased progressively from 32 to 75 mg/100 ml within two weeks.

In this case too the kidney history was sparse. The patient had only two previous episodes of urinary tract infections. The blood pressure was 140/70. The teeth were opaque and showed diffuse brownish mottling. The urine showed proteinuria (grade 3) but good ability to concentrate. It contained occasional red and white blood cells; hyaline and granual casts. Inulin clearance was reduced to 19 ml/min., para-aminohippurate clearance to 99 ml/min. and creatinine clearance to 17 ml/min.

The urogram showed marked reduction in the size of the kidney, blunting of its calyces and widening of the kidney pelvis of the ureter. Both ureteral orifices were incompetent. The hemoglobin was low (8.8 gm/100 ml), blood urea 133 mg/100 ml; serum calcium 8.9 mg/100 ml; phosphorus, 5.9 mg/100 ml.

X-rays of the lumbar spine showed blurring coarse trabeculation of the vertebrae and pelvic bones. There was evidence of systemic acidosis. The patient had a bilateral ureteroneocystostomy. However the improvement was not remarkable. The patient continued to have nocturia, polydipsia, and polyuria (approximately 4 liters/day).

In their comments the authors point to the efficient clearance of fluoride through the kidneys. The diagnosis systemic fluorosis was established by the dental and roentgenographic bone changes. In neither patient could the dental changes be attributed to tetracycline as neither patient had taken the drug.

The authors believed that kidney disease is not likely to have resulted from fluoride intake. They also stated that according to most authors fluoride levels of 4 ppm or more are necessary to induce systemic fluorosis. [In one of the 3 papers cited in support of the latter statement (Azar, H. A., et al., Ann. Intern. Med., 55:193-200, 1961) the water contained between 0.8 to 3.45 ppm. Ed.]

G.L.W.

FERTILITY IMPAIRMENT IN MICE ON A LOW FLUORIDE INTAKE

by

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(Abstracted from Science, 177:893, 1972)

The authors report a decreased fertility rate as well as a delayed onset of sexual maturity in female mice fed a low fluoride diet.

Female albino mice were divided into two groups and were given either deionized water (58 mice) or deionized water containing 50 parts per million of sodium fluoride (55 mice). Both groups were fed a low fluoride diet containing 0.1 to 0.3 ppm of fluoride. The composition and method of preparation was not recorded.

The mice were mated at 8 weeks of age and litter production was observed over a 25 week period to a maximum of four litters. Litter production was also assessed in second generation females taken primarily from the fourth litters of the first generation mice. These mice were maintained on the same fluoride intake as their mothers.

The mean age at which mice gave birth to their first litter was not significantly different from 13 weeks for both generations of the high fluoride group and for the first generation of the low fluoride group. However, a highly significant delay in the birth of the first litter occurred in the second generation animals on the low fluoride intake. Mice in the low fluoride group showed a progressive impairment in reproductive capcity. All animals of the first generation produced one litter, but progressively fewer mice gave birth to additional litters and less than 50% produced four litters. The decrease in reproduction was more pronounced in the second generation mice on the low fluoride intake. No differences were found between the high and low fluoride groups in the weight of the mice at birth or at five days, or in the number of mice per litter.

The ability of added fluoride to restore normal fertility to mice previously rendered subfertile on a low fluoride intake was also investigated. Female mice of demonstrated impaired fertility were divided into two groups -- half were given a diet of 50 ppm of fluoride and half were retained on the low fluoride intake. Again litter production was assessed over 20 weeks. Mice retained on the low fluoride intake continued to show impaired reproduction whereas the mice transferred to the high fluoride intake showed an improvement in litter production.

The basis for the low fertility in mice receiving a low fluoride intake was not determined, but to the authors this study demonstrated that fluorine is an essential trace element.

K.J.

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EFFECT OF SODIUM FLUORIDE ON THE PLACENTA IN THE RAT

by

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(Abstracted from the Arch. Oral Biol., 17:371-74, 1972)

The authors studied the effect of fluoride injected intraperitoneally and subcutaneously into rats between the 10th and 18th day of pregnancy. The daily doses were 1, 5, 10, 15 or 20 mg/kg body weight of sodium fluoride diluted in saline solution. Five groups of 6 rats each received the injections subcutaneously, five other groups intraperitoneally; another group served as control.

All rats were killed on the 18th day of pregnancy and the heads of the fetuses as well as the placentae were studied histologically and microscopically. In all fluoride-treated groups, higher percentages of intrauterine dead fetuses were found and the placentae were necrotic. The differences in percentage of dead fetuses and necrotic placentae between the fluoride and the saline treated rats were highly significant. The viable fetuses showed no maxillo-facial malformations. A marked post-mortem autolysis was noted in the dead fetuses, in the intrauterine dead and in the necrotic placentae. The placentae of the viable fetuses and of those dead fetuses which were not necrotic, showed no pathology.

The fact that every intrauterine dead fatality was associated with a necrotic placenta and that no normal placentae were found whenever there was a fetal death, suggested to the authors that the toxic action of fluoride takes place in the placenta and that the fetal mortality resulted from placental damage. The authors believe that the placenta acts as a partial barrier to fluoride. They warn however that one must be cautious in applying these results to humans because the human placenta is different from that of the rat and the dog.

G. L. W.

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ACUTE SODIUM FLUORIDE POISONING

by

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(Abstracted from the Journ. Amer. Med. Assoc., 222:816-17, 1972)

The authors described the case of a 25 year-old Negro who had ingested 120 gm of roach powder (97% sodium fluoride) in a suicide attempt. This case is remarkable because it is probably the largest dose of the drug ever ingested by man without a fatal outcome. (In former fatal cases the dose has ranged from about 1 to 5 gms - Ed.). Furthermore the treatment instituted is undoubtedly the most complete and effective ever reported.

The patient experienced extensive nausea and vomiting as well as excessive salivation for approximately one hour prior to admission to the hospital where the following measures were instituted:

The stomach was immediately lavaged with lime water (0.15% calcium hydroxide) and 1 gm of gluconate calcium was given intravenously. One hour later, tetanic contractions occurred followed by respiratory arrest and ventricular fibrillation. This prompted endotracheal intubation, external cardiac massage, and intravenous administration of gluconate calcium (2 gm) and magnesium sulfate (8 mEq). During the next 12 hours, direct-current countershock was applied 63 times to reverse episodes of ventricular fibrillation. On two occasions cardiac standstill responded to epinephrine given intravenously. The patient received lidocaine at 5 mg/min episodes but the ventricular fibrillation continued until an intravenous atrial pacemaker was inserted in an attempt to "overdrive" the irritable ventricular focus. Subsequently intermittent signs of tetany were controlled by additional injections of calcium (4 gms in 12 hours), and 16 milliequivalents of magnesium as magnesium sulfate intravenously. At one time a generalized convulsion was treated with 10 mg intravenous diazepam, a tranquilizer.

During the following 4 weeks the electrocardiogram revealed a pattern consistent with an acute anterior myocardial infarction. The patient was given lidocaine to reduce cardiac irritability, fluids and diuretics (mannitol and furosemide) all intravenously.

The authors considered the intravenous injections of calcium and lidocaine most effective treatment for the control of the ventricular fibrillation.

After 12 hours the patient was able to respond to verbal questions, but

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was unable to swallow and regurgitated through the nose. Complete blockage of the pharyngoesophageal junction found by X-ray necessitated feeding through a gastrostomy for about 30 days. Subsequently the swallowing became normal.

With respect to laboratory findings the initial serum fluoride concentration was 2 micrograms/ml. Arterial pH and blood gases revealed a combination of respiratory and metabolic acidosis, which was corrected by administration of 610 mEq of sodium bicarbonate intravenously during the first 12 hours.

The serum lactic acid dehydrogenase (LDH) was 2,825 μU/ml. There was also a high serum uric acid level (11.0 mg/100 ml); however the BUN and the creatinine clearance were consistently normal and a slight urinary protein excretion cleared on the third hospital day. The serum calcium ranged from 6.5 to 9.5 and serum phosphorus from 0.8 to 3.4.

The authors attributed the cardiac and central nervous system manifestations to hypocalcemia, the presence of hemorrhagic gastroenteritis to the formation of hydrofluoric acid which produced superficial and deep ulcerations of the esophagus, of the oral mucous membranes and of the skin. Susceptibility to hemorrhages was ascribed to prolongation of prothrombin time. Hypokalemia and hypomagnesemia which can contribute to myocardial irritability, could have induced ventricular fibrillation. The severe acidosis was linked to the formation of lactic acid.

G. L. W.

CORRESPONDENCE

To the Editor:

I appreciate your note on the editorial comments with respect to the fluoridation paper by Geever et al. (HSMHA Health Reports, 86:820-7, 1971). Members of the staff of the National Institute of Dental Research have assisted me in preparing a reply to your editorial remarks.

1. You state that the specimens from New York and Albany do not constitute suitable controls to those from the fluoridated area of Grand Rapids; that the level of fluoride found in the bones is too high to be accounted for by water intake alone; and that the 'control' fluoride levels in bones were in the range of those seen in fluorotic bones of other studies. In response we would say that while other sources of fluoride intake were not studied in the present report, the bone levels of fluoride in the specimens from Grand Rapids were significantly higher than those found in samples from the control areas; that in neither the experimental nor the control bone specimens was there found any evidence of fluorosis; and that with regard to the uptake of fluoride in both cases, it appears to follow the 'plateau' results observed in other studies as a function of age--that is, there was no suggestion of an unusual pattern of fluoride incorporation into bone.

2. Although the investigators did not report mineral analyses of the water in any of the three cities nor did they study the concentrations of phosphorus and other elements in the diet which could conceivably affect fluoride uptake, the results of the present study are in good agreement with others cited by the authors with respect to the observation that there were no significant skeletal differences between the bones of two groups, one of which had significantly higher levels of fluoride than the other. In other words, data on other constituents in water and diet may not be relevant to the purpose of this study.

3. Exclusion of cases of parathyroid and certain types of renal disease, as well as examples of other illnesses, was mandatory in view of the extensive effects upon bone structure caused by these conditions. In addition, there is no evidence to show that such maladies are related to fluoride

intake, especially in the concentrations dealt with in the present study.

4. You feel that the fluoride levels in the control aortas are 'high' and emphasize that the finding of 2340 ppm in the aorta of a young person (under 20 years) cannot be explained by pointing to the propensity of aortic and other soft tissue to calcify; that calcification of aortas is often a manifestation of skeletal fluorosis; and that it is an important possibility that intake of fluoridated water may damage blood vessels. As I am sure you appreciate, it is well known that calcium begins to accumulate in aortas after the age of 20. Contrary to the editor's position, it has been shown that fluoride accumulates as an adventitious constituent in the mineral phase during calcification of both hard and soft tissues. While the aorta of a single individual from the control region below the age of 20 years contained 2340 ppm of fluoride, histopathological examination of all the specimens did not show any relationship between atherosclerosis in the abdominal aorta and intake of fluoridated water. Again, none of the cases were reported to show evidence of fluorosis.

5. The exact duration of residence for each subject was often difficult to obtain. Unpublished clinical data were obtained in each case but, as was stated in the article, those subjects were selected whose deaths occurred in such a way as not to obscure any possible effects of long-term fluoridated water intake upon skeletal changes.

We would agree with the final points made. However, it should be emphasized again that no evidence of fluorosis was observed in any of the specimens irrespective of the residences of the subjects.

Our conclusion is that the study under discussion should be considered as an addition to the large and increasing body of evidence that long-term intake of fluoridated waters (1 ppm concentration) does not have deleterious effects upon health.

Also we would agree that no evidence has been developed here to support the idea that fluoride is beneficial in treating osteoporosis or in preventing atherosclerosis.

Signed Jesse L. Steinfeld, M. D.
Surgeon General

EDITOR'S COMMENT

Surgeon General Steinfeld emphasizes repeatedly (in paragraphs 1, 2, 3, and 6) that no evidence of skeletal fluorosis was found in any of the specimens (HSMHA Health Reports, 88:820-28, 1971). Such evidence should not be anticipated because "cases of parathyroid and certain types of renal disease were excluded from the study." The categorical statement that these maladies are not related to fluoride intake is not borne out by the available literature. But even if Dr. Steinfeld were correct and the bone changes of parathyroid and certain kidney diseases were not related to fluoride it is impossible to differentiate unequivocably between skeletal changes of fluorosis and those of kidney and parathyroid disease in order to exclude the latter from the survey.

Dr. Steinfeld's letter acknowledges the numerous other deficiencies pointed out in FLUORIDE, 5:231-2, 1972: lack of data concerned with the mineral composition of the respective water supplies; disregard of fluoride uptake from sources other than drinking water (particularly in such polluted urban areas as New York City) failure to determine the exact duration of residence for each subject; disregard of the nonskeletal phase of fluorosis and of individual cases which show deviations from normal.

Accumulation of fluoride "as an adventitious constituent" in both hard and soft tissue is extremely rare in individuals 20 years of age. Such observations should have been subject to special studies to determine whether or not the arterial calcification was related to their high fluoride content.

The above deficiencies characterize most, if not all, studies designated by Dr. Steinfeld as "the large and increasing body of evidence" which purport to prove the safety of fluoridation. A reexamination of the official surveys in the light of recent advances in our knowledge is urgently needed.

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