Urinary D-Glucaric Acid Excretion in the Seveso Area, Polluted by Tetrachloro-dibenzo-*p*-dioxin (TCDD): Five Years of Experience

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On July 10, 1976, an explosion in a factory in Seveso, Italy, located 30 km north of Milan, producing trichlorophenol caused the release of TCDD-containing compounds in the surrounding area. Since extremely small doses of TCDD have been shown to induce hepatic microsomal enzymes in animals, urinary D-glucaric acid excretion (a measurable index of enzyme induction), has been investigated in Seveso in adults and children 6 to 8 years old, in order to clarify whether levels of environmental exposure to TCDD were sufficient to produce an induction in man. Urine samples were collected from 1976 to 1981. As a control group, people living in Cannero (a nonindustrialized village on lake Magiore), in Busto Arsizio (a small industrial town near Milan) and in Lentate (a noncontaminated zone near Seveso) were chosen. In the first period of collection, children with chloracne (which is considered to be a characteristic manifestation of intoxication with chlorinated products) showed significantly increased levels of D-glucaric acid excretion compared to children without chloracne living in the same zone.

As far as chronic exposure is concerned, up to 3 years after the accident both adults and children living in the Seveso area showed a statistically significant enhancement of D-glucaric acid elimination compared to the control groups.

This study demonstrates that adults and children living in the polluted zones had an increased activity of hepatic microsomal enzymes for some years, since, although the urinary excretion of D-glucaric acid is only an indirect measure of enzyme activity, studies in man have indicated that it is, however, sensitive and quantitative. As far as the cause of this enhancement is concerned, since at least in children it is possible to exclude the influence of alcohol, contraceptives or other drugs, it is reasonable to conclude that tetrachlorodibenzo-*p*-dioxin (TCDD), an inducing agent, could be responsible for this phenomenon.

Shortly after noon (12:40) on July 10, 1976, in Seveso, Italy (located 30 km north of Milan), a reaction at the Icmesa chemical plant producing trichlorophenol got out of control, and an explosion spread substances to the surrounding area one of which was tetrachloro dibenzo-*p*-dioxin (TCDD).

TCDD is one of the most toxic synthetic chemicals yet discovered, and there are large differences in species susceptibility. Many organs and systems were demonstrated to be affected after TCDD intoxication (*1–3*).

A few days after the accident in Seveso, particularly in the area near the Icmesa plant, some people, especially children, developed skin lesions of various kinds, including chloracne, which is considered to be a characteristic, though nonspecific, manifestation of intoxication with chlorinated products (*4–8*).

The contaminated area was divided into three zones,

depending on the TCDD concentration of the soil (Table 1). The inhabitants of zone A were evacuated 15 days after the explosion to areas without detectable TCDD, whereas those of zones B and R continued to live in their homes.

In laboratory animals TCDD is known to cause hypertrophy of the smooth endoplasmic reticulum and to induce microsomal enzymes in the liver at doses lower than those that damage various organs (*9–26*).

In order to clarify whether there was an increased number of persons with induced hepatic enzymes among the Seveso population, we assayed urinary D-glucaric

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Table 1. TCDD-contaminated territory.

	Zone A	Zone B	Zone R
Area, km²	0.73	2.52	12
Inhabitants	736ᵃ	4699	31,800
TCDD in soil, μg/m²	51–5477	5–50	<5

ᵃAll evacuated.

Table 2. Case material.

	Number
Adults: Chronic exposure	
(urines collected in February 1978)	
Seveso (zone B)	117
Cannero (controls)	127
Children: Acute exposure	
(urines collected in August–December 1976)	
Subjects with chloracne (zone A)	14
Subjects without chloracne (zone A)	17
Children: Chronic exposure	
(urines collected in February 1979)	
Zone A (evacuees)	16
Zone B	51
Busto Arsizio (controls)	60
Cannero (controls)	26
Children: Chronic exposure	
(urines collected in May 1981)	
Zone A (evacuees)	34
Zone B	61
Zone R (Mulinello of Cesano)	62
Zone R (Polo of Meda)	59
Lentate (controls)	61

acid, which is considered to be a convenient index, because although indirect, it is simple, sensitive, reproducible and correlates quantitatively with the degree of enzyme induction (27–38).

Various groups of people (adults and especially children, with ages between 6 and 8 years) were studied in different periods; the results of the follow-up of the population, as far as urinary D-glucaric acid excretion is concerned, is presented in this paper. Preliminary results concerning only the children were presented elsewhere (39).

Materials and Methods

The case material is summarized in Table 2. As far as the selection of the subjects is concerned, some considerations are necessary.

Adults 1978

For one week, urine of people living in zone B who were called to undergo blood tests, according to the monitoring plan for Seveso, were collected. The compliance with the request was about 80%. As a control group we collected urine samples from adults living in Cannero (a small town on Lake Maggiore, 130 km from Milan, in a nonindustrialized area, uncontaminated by environmental toxin). Half of the population of this town was selected (450 people) and was asked to undergo a clinical visit, plus urine and blood examina tions. The compliance with our request was about 90%. One-third of the collected samples, chosen at random, were utilized for D-glucaric acid estimation. Both in Seveso and in Cannero, people affected by overt renal or liver disease or taking liver enzyme-inducing agents were excluded.

Children 1976

This retrospective study was concerned with children known to have been in contact with the poison because they presented with chloracne. The levels of D-glucaric acid in their urine samples collected in the period August–December 1976 and immediately frozen at –30°C were compared with those of children of the same zone without skin lesions, whose urine was collected and frozen in the same period. Sixty names were drawn: 30 children with skin lesions and 30 without skin lesions. Of these it was possible to recover from thousands of test tubes frozen at –30°C 31 samples, i.e., 14 subjects (9 girls, 5 boys) with chloracne and 17 subjects (10 girls, 7 boys) without any skin lesions.

Children 1979

Urine samples of children living in zone B and in zone A (evacuees) were collected. In some of these children, D-glucaric acid excretion was also estimated one year later. As control groups we chose children living in Cannero and Busto Arsizio (a town about 30 km from Milan, in a highly industrialized area but not contaminated with TCDD).

Children 1981

In May 1981 we compared urinary excretion of D-glucaric acid in children of zone A, zone B, two groups living in zone R and as a control, children living in Lentate, a zone near Seveso not contaminated by TCDD.

All the urine samples were collected, after informed consent, from pupils in the first three elementary school grades; the compliance with our request was about 90%. The urine samples were assayed for D-glucaric acid by the method of Simmons et al. (40) and corrected for variations in urine concentrations from the creatinine values. Because in children a portion of creatine is not converted into creatinine, we expressed the urinary D-glucaric acid values in relation to the sum of the creatine converted into creatinine and the creatinine itself.

For the assay of urinary creatinine we used the method of Jaffé without deproteinization, as standardized by Roche Products (41). Creatine was converted into creatinine by acidification and boiling of the urine for 4 hr (40). The values found are then expressed in μmole saccharo-1,4-lactone/g urinary creatinine.

Statistical Methods

The relatively small sizes of the samples analyzed were not suitable for a proper analysis of the distributions of the values of D-glucaric acid in urine samples. However, these distributions appeared to be positively skewed and rather irregular; moreover large differ ences in their dispersion parameters were observed. Therefore, it seemed sensible to process data accorà:.-- to distribution-free methods.

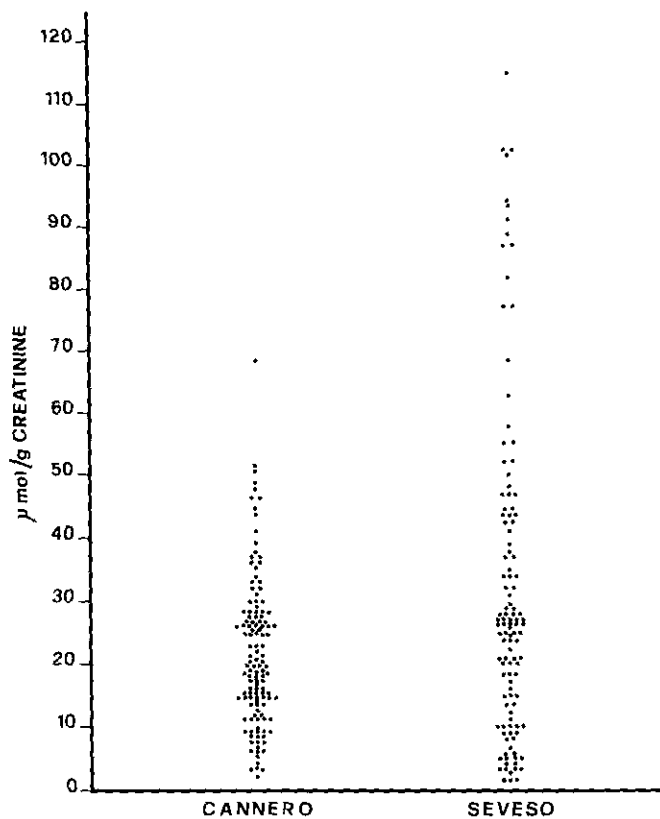Results obtained in 1976 regarding children with _ without chloracne were compared by using Krush.

FIGURE 1. Urinary ᴅ-glucaric acid levels in adults living in Seveso (zone B) and Cannero (controls) and collected in February 1978.
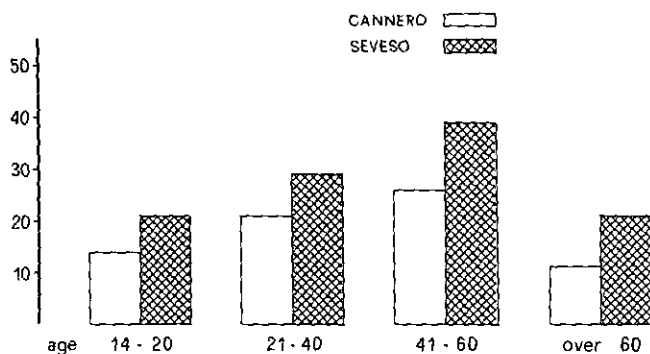

FIGURE 2. Urinary ᴅ-glucaric acid values related to age.

FIGURE 3. Urinary ᴅ-glucaric acid excretion in urines collected from children in the period August–December 1976 (acute exposure).

Wallis (43) nonparametric analysis of variance. The same method was followed to analyze data collected in 1979 from exposed subjects (Seveso: zones A and B) and controls (Cannero and Busto Arsizio). Dunn's techniques (44) were adopted to perform multiple comparisons. The difference between the results obtained in 1979 and in 1980 was tested according to Wilcoxon (45), while the association between the same results was measured by Spearman's rank correlation index (46).

Results

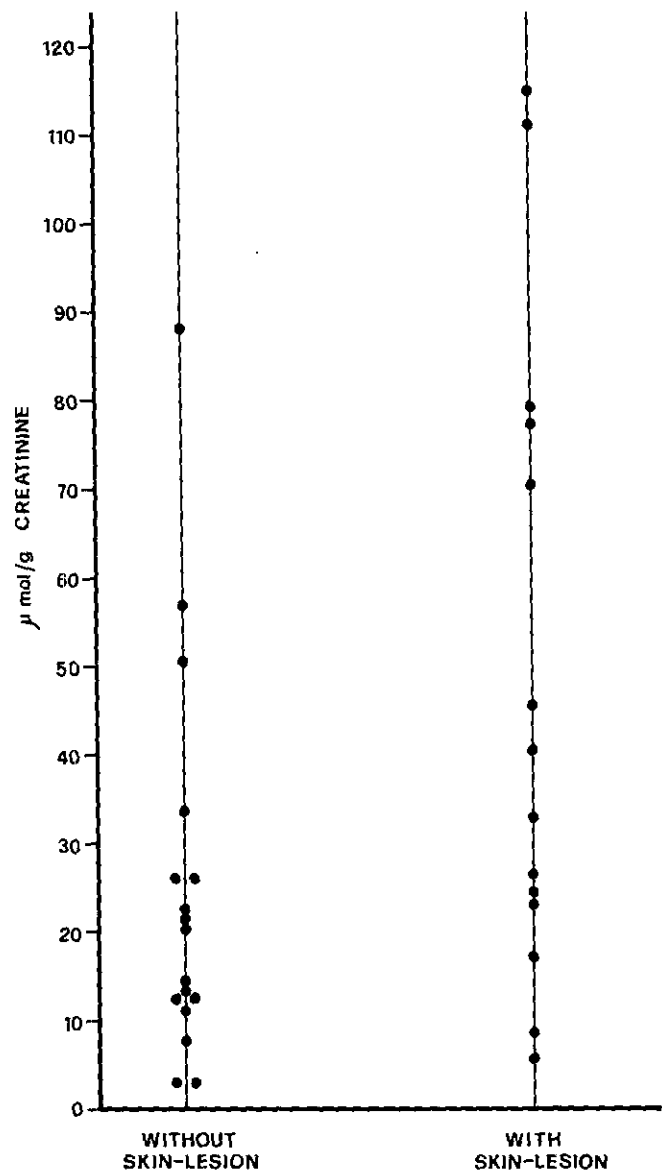The ᴅ-glucaric acid excretion in urines of adults collected in 1978 was higher in people living in the Seveso area than in those of Cannero, the difference being statistically significant (median 27.1 µmole/g of creatinine in Seveso and 19.8 in Cannero; lower quartile, 11.9 and 14.8, respectively; upper quartile, 42.1 and 29.3, respectively; $p < 0.05$) (Fig. 1). The distribution of the values related to sex was similar, while significant dependence of ᴅ-glucaric acid levels on age in both centers was found: in all ages exposed subjects showed excretion significantly higher than controls ($p < 0.05$) (Fig. 2).

Figure 3 shows the ᴅ-glucaric acid levels in urine samples collected from children in August–December 1976 and could represent the effect of an acute exposure. The median is 20.5 in children without skin lesions and 39 in those with skin lesions (lower quartile 11.8 and 23.3, respectively; upper quartile, 29.9 and 77.1, re-
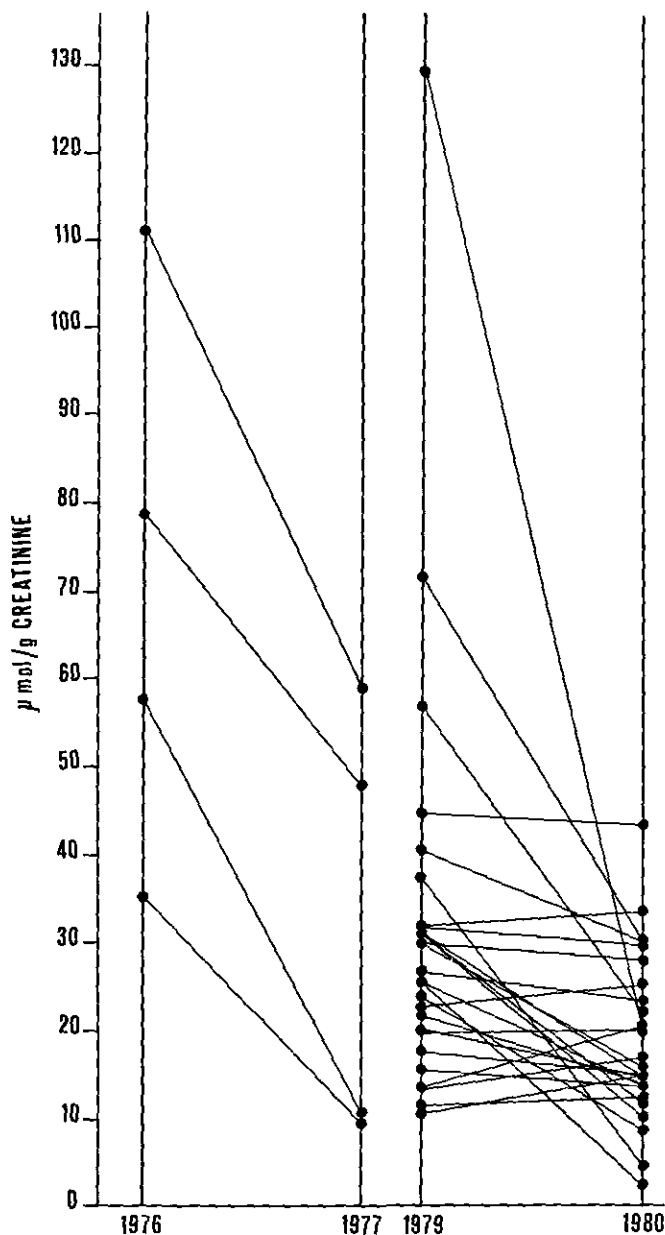
FIGURE 4. Comparison between D-glucaric excretion in samples of children collected in two different periods of time. Four subjects (left of the figure) were living in zone A, the others in zone B.

FIGURE 5. Urinary D-glucaric acid excretion in children living in Cannero, Busto Arsizio, in zone A (evacuees) and zone B of Seveso and collected in the period February–March 1979 (chronic exposure).

Table 3. Levels of D-glucaric acid in urines of 27 children living in zone B: comparison between values observed in 1979 and 1980.

	Seveso (zone B) 1979	Seveso (zone B) 1980	Wilcoxon test	Spearman rank correlation coefficient
Median	26.8	17.0	2.93[a]	0.427[b]
Lower quartile	19.4	13.5		
Upper quartile	31.7	26.8		

[a]Wilcoxon index is normally distributed: children under study showed in 1979 D-glucaric acid levels significantly higher than in 1980 ($p \leq 0.01$).

[b]D-Glucaric acid levels measured in 1979 are significantly correlated to those observed in 1980 ($p \leq 0.01$).

spectively). The difference is statistically significant ($p < 0.05$). In four of the children of zone A who left the zone 16 days after the explosion, we measured D-glucaric acid excretion in two samples, the first collected in August–September 1976, the second in February–March 1977. In all cases lower amounts were observed in the latter sample (79–49, 51–11, 36–10, 111–59 μmole/g of creatinine) (Fig. 4).

Levels of D-glucaric acid in urine samples collected in children in March 1979 are shown in Figure 5. Statistical analysis demonstrated that there was no difference between the children of Busto Arsizio and Cannero; the
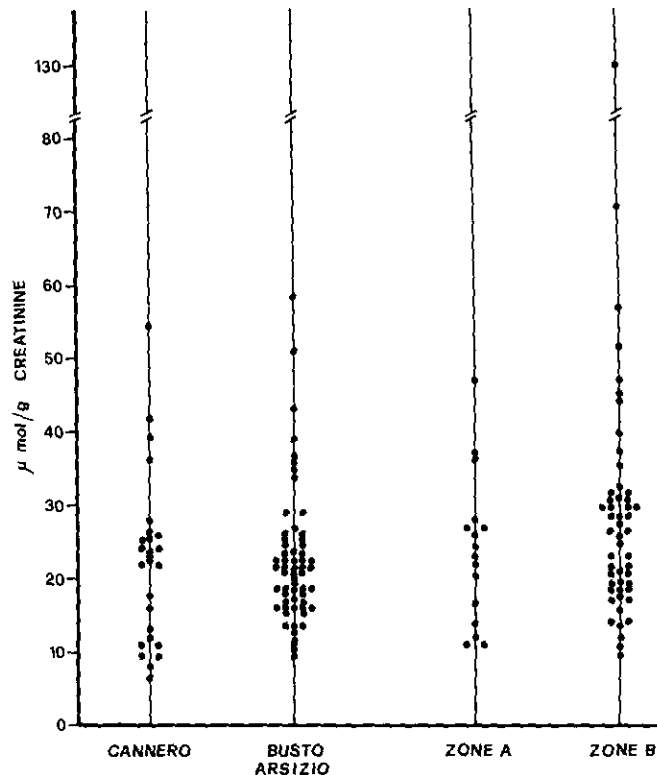
two groups were therefore used as a single control group in the subsequent comparison (86 subjects overall, median 21.8, lower quartile 16, upper quartile 25.5). Of the children exposed to TCDD, D-glucaric acid concentration in zone A (evacuees) was similar to that in controls (median 23.2, lower quartile 15, upper quartile 27.2); however, in zone B the excretion was significantly higher (median 26 μmole/g of creatinine, lower quartile 19.1, upper quartile 31.5, $p < 0.05$). The comparison between D-glucaric acid values observed in 1979 and 1980 in urines of children living in zone B showed significantly lower levels in 1980 (Fig. 4 and Table 3).
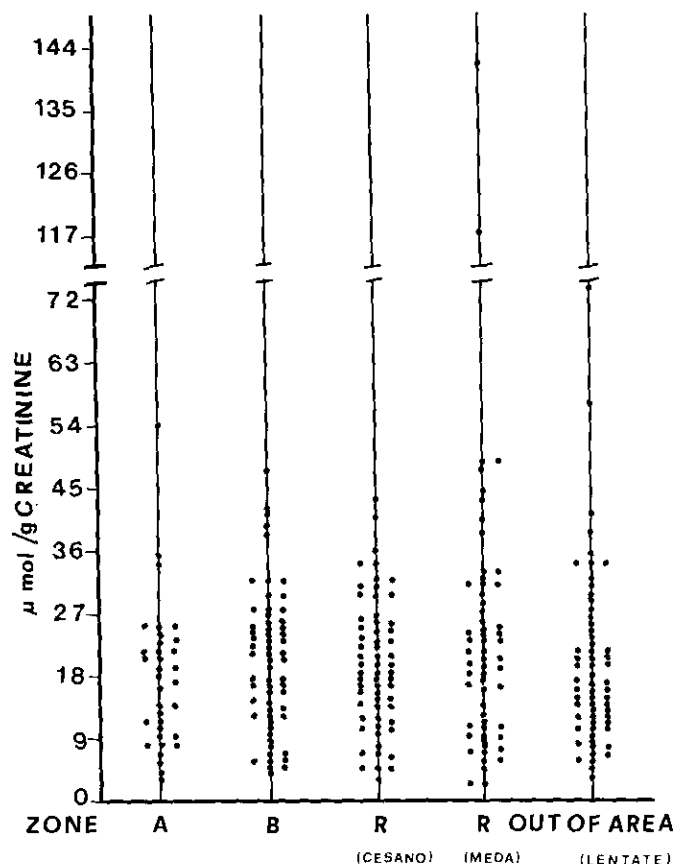
FIGURE 6. Urinary D-glucaric acid excretion in children living in different zones of Seveso and in Lentate (controls). These samples were collected in May 1981.

Table 4. D-Glucaric acid levels in the urines collected in 1981.

	Controls (Lentate)	Zone A	Zone B	Zone R (Mulinello)	Zone R (Polo)
No. of children	61	34	61	62	59
D-Glucaric acid levels, μmole/g					
Median	17.2	16.7	19.8[a]	17.8	20.1[a]
Lower quartile	10.6	8.2	12.1	11.4	10.1
Upper quartile	23.0	22.4	23.2	23.2	28.7

[a]Kruskal-Wallis test: $p = 0.05$.

Finally Figure 6 and Table 4 show the D-glucaric acid excretion in urine samples collected in children in May 1981. A trend toward an increased excretion in children living in zone B and in zone R (Polo) was observed; however it does not reach a significant difference with respect to the controls $(0.10 > p > 0.05)$.

Discussion

Our study could be divided into two parts. First D-glucaric acid excretion in children with and without chloracne who lived in the most contaminated zone before the evacuation was evaluated. The urine samples

were collected in the period August–December 1976, and this could be considered an acute exposure. Chloracneic children had significantly higher urinary D-glucaric acid concentrations than nonchloracneic children from the same zone. It is likely that the Icmesa disaster, responsible for the skin lesion, was also the cause of this change. As far as the responsible substance is concerned, it is only possible to suppose that among the substances released that day (glycols, caustic soda, trichlorophenol and TCDD), the latter is the most indicated, since only this has been demonstrated to be a potent inducer (9–26).

In the second part of the study, estimation of D-glucaric acid excretion was performed in adults and children living in the polluted zones; since the research was carried out 2, 3, 4 and 5 years after the explosion, this could be considered a chronic exposure.

Up until 1979 adults and children living in the Seveso area were demonstrated to have significantly higher values of D-glucaric acid excretion, in comparison to those observed in controls (inhabitants of Cannero and Busto Arsizio).

Because in children factors such as alcohol, contraceptives or other drugs commonly known to act as enzyme inducers can be ruled out as a cause for the increase in excretion, it is reasonable to surmise that in this, as in the other study, TCDD is responsible for the phenomenon.

It is hardly conceivable that the differences observed could have been due to different lifestyles or eating habits and as for nonspecific environmental pollution or the like, since the urinary D-glucaric acid excretion values in the children of Cannero, a lakeside village, and in those of Busto Arsizio, a small but industrialized and heavily polluted town, were practically identical.

In zone B, TCDD (but not the other compounds released in the toxic cloud, which were quickly broken down) has been present in the soil for years, and children could have easily come into contact with it.

The similar elimination of D-glucaric acid values between children of zone A and controls can be explained by the fact that children from zone A were moved away from the polluted zones a few days after the accident, and it is likely that 3 years are sufficient for complete elimination of any poison absorbed in July 1976.

From 1980 the elimination of D-glucaric acid was observed to return toward the normal value.

The lower level observed in 1980 compared with 1979 in the majority of children living in zone B could be explained either by major precautions taken by the children whose parents were advised of the results of the test and of its significance, or by a reduction of TCDD present in the soil (as a consequence of the natural degradation and the decontamination).

The almost similar level of D-glucaric acid observed in 1981 in children living in different zones of Seveso and in Lentate (uncontaminated town near the Icmesa plant) supports the latter hypothesis.

In conclusion, determination of urinary D-glucaric acid, a sensitive index for hepatic microsomal enzyme induction, enabled us to prove the existence of induction in subjects living in the TCDD-polluted zones.

Our findings also show that D-glucaric acid estimation could be a useful method for detecting metabolic changes or adaptation, such as enzyme induction, which precede liver alteration caused by poisons like TCDD, even in the absence of clinical disease.

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