Urinary D-Glucaric Acid Excretion in the Seveso Area, Polluted by Tetrachlorodibenzo-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 Canero (a nonindustrialized village on Lake Maggiore), 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 Icema 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 Icema 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

| Table 1. TCDD-contaminated territory |
|-------------------------------------|
| Zone A   | Zone B   | Zone R   |
| Area, km² | 0.73     | 2.52     | 12       |
| Inhabitants | 736*     | 4699     | 31,800   |
| TCDD in soil, μg/m² | 51-5477* | 5-50     | <5       |

*All evacuated.
Table 2. Case material.

| Adults: Chronic exposure | Number |
|-------------------------|--------|
| (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 examinations. 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 differences in their dispersion parameters were observed. Therefore, it seemed sensible to process data according to distribution-free methods.

Results obtained in 1976 regarding children with and without chloracne were compared by using Kruskal-
URINARY D-GLUCARIC ACID EXCRETION

120
110
100
90
80
70
60
50
40
30
20
10
0

\[ \text{\( \mu \text{mol/g CREATININE} \)} \]

\begin{tabular}{|c|c|c|}
\hline
\text{CANNERO} & \text{SEVESO} \\
\hline
\text{14 - 20} & \text{30 - 40} & \text{41 - 60} & \text{over 60} \\
\hline
\end{tabular}

\textbf{FIGURE 2.} Urinary D-glucaric acid values related to age.

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).

\textbf{Results}

The D-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 \( \mu \text{mole/g of creatinine} \) 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 D-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 D-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-
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).

**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) | Wilcoxon test | Spearman rank correlation coefficient |
|-----------|-----------------|---------------|---------------------------------------|
| Median    | 26.8            | 17.0          | 2.93*                                 |
| Lower quartile | 19.4          | 13.5          |                                      |
| Upper quartile | 31.7          | 26.8          | 0.427*                                |

*Wilcoxon index is normally distributed: children under study showed in 1979 d-glucaric acid levels significantly higher than in 1980 ($p < 0.01$).

*D-Glucaric acid levels measured in 1979 are significantly correlated to those observed in 1980 ($p < 0.01$).
URINARY D-GLUCARIC ACID EXCRETION

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.

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

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).

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

| Zone A | Zone B | Zone R (Mulinello) | Zone R (Polo) |
|--------|--------|--------------------|---------------|
| Controls (Lentate) | 61 | 34 | 61 | 62 | 59 |
| D-Glucaric acid levels, µmole/g | 17.2 | 16.7 | 19.8* | 17.8 | 20.1* |
| Median | 10.6 | 8.2 | 12.1 | 11.4 | 10.1 |
| Lower quartile | 12.0 | 22.4 | 23.2 | 23.2 | 28.7 |

*Kruskal-Wallis test: p = 0.05.

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.
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.

REFERENCES

1. Greig, J. B., Jones, G. Butler, W. S., and Barnes, J. M. Toxic effect of TCDD. Food Cosmetic Toxicol. 11: 585–595 (1973).
2. Schewtz, B. A., Norris, J. M., Sparschu, G. L., Rowe, V. K., Gehring, P. J., Emerson, J. L., and Gerbig, C. G. Toxicology of chlorinated dibenzo-p-dioxin. Environ. Health Perspect. 5: 87–99 (1970).
3. Case, A. A. TCDD—clinical aspects of poisoning. Clin. Toxicol. 9: 963–967 (1976).
4. Telegina, K. A., Bikbulatova, L. I. Affection of the follicular apparatus of the skin in workers employed in the production of butyl ether of 2,4,5-trichlorophenoxyacetic acid. Vestn. Dermatol. Venereol. 44: 55–59 (1970).
5. Poland, A. P., Smith, D., Metter, G., and Possick, P. A. health survey of workers in 2,4-D and 2,4,5-T plant with special attention to chloracne, porphyria cutanea tarda and psychologic parameters. Arch. Environ. Health 22: 316–327 (1971).
6. Goldmann, F. J. Schwerverste akute Chlorakne durch Trichlorphenol-Zersetzungprodukte. Arbeits-med. Sozialmed. Arbeits tyg., 7: 12–18 (1972).
7. May G. Chloracne from accidental production of tetrachlorodibenzo-dioxin. Brit. J. Ind. Med. 30: 276–283 (1973).
8. Taylor, J. S., Wuthrich, R. C., Lloyd, K. M., and Poland, A. Chloracne from manufacture of a new herbicide. Arch. Dermatol. 113: 616–619 (1977).
9. Greig, J. B. Effect of 2,3,7,8-tetrachlorodibenzo-1,4-dioxin on drug metabolism in the rat. Biochem. Pharmacol. 21: 3196–3198 (1972).
10. Greig, J. B., and De Matteis, F. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on drug metabolism and hepatic microsomes of rats and mice. Environ. Health Perspect. 5: 211–219, 1973.
11. Lucier, G. W., McDaniel, O. S., Hook, G. E. R., Fowler, B. A., Sonawane, B. R., and Faeder, J. L. TCDD-induced changes in rat liver microsomal enzymes. Environ. Health Perspect. 5: 99–209 (1973).
12. Lucier, G. W., McDaniel O. S., Fowler, B. A., Sonawane, B. R., Falez, E., and Hook, G. E. R. TCDD-induced changes in rat liver microsomal enzymes. Environ. Health Perspect. 5: 199–209 (1973).
13. Poland, A., and Glover, E. Chlorinated dibenzo-p-dioxins: potent inducers of δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase. II. A study of the structure-activity relationships. Mol. Pharmacol. 9: 1320–1334 (1973).
14. Poland, A., and Glover, E. Comparison of 2,3,7,8-tetrachlorodibenzo-p-dioxin, a potent inducer of arylhydrocarbonhydroxylase, with 3-methylcholanthrene. Mol. Pharmacol. 10: 249–259 (1974).
15. Lucier, G. W., McDaniel, O. S., and Hook, G. E. R. Nature of the enhancement of hepatic uridine-diphosphate-glucuronyltransferase activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Biochem. Pharmacol. 24: 325–334 (1975).
16. Hook, G. E. R., Haseman, K. J., and Lucier, G. W. Induction and suppression of hepatic and extrahepatic microsomal foreign compound-metabolizing enzyme systems by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Chem.-Biol. Interact. 10: 199–214 (1975).
17. Lucier, G. W., Sonawane, B. R., McDaniel, O. S., et al. Postnatal stimulation of hepatic microsomal enzymes following administration of TCDD to pregnant rats. Chem.-Biol. Interactions 11: 15–26 (1975).
18. Beatty, P. W., and Neal R. A. Induction of DT-diaphorase by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Biochem. Biophys. Res. Comm. 68: 197–204 (1979).
19. Berry, D. D., Zachariah, P. K., Namkung, M. J., McDaniel, O. S., and Hook, G. E R. Transplacental induction of carcinogenhydroxylation system with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 36: 569–584 (1976).
20. Alito, A., and Parkki, G. Organ specific induction of drug metabolizing enzymes by 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. Toxicol. Appl. Pharmacol. 44: 107–114 (1978).
21. Goldstein, J. A., Friesen, M., Scotti, T. M., Hickman, P., Hass, J. R., and Bergman, H. Assessment of the contribution of chlorinated dibenzo-p-dioxins and dibenzo furans to hexachlorobenzene-induced toxicity, porphyria, changes in mixed function oxygenases and histopathological changes. Toxicol. Appl. Pharmacol. 46: 633–649 (1978).
22. Kitchin, K. T., and Woods, J. S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin induction of aryl-hydrocarbon hydroxylase in female rat liver. Evidence for the de novo synthesis of cytochrome P-448. Molec. Pharmacol. 14: 890–899 (1978).
23. Norman, R. L., Johnson, E. F., and Muller-Eberhard, U. Identification of the major cytochrome P-450 form transplacentally induced in neonatal rabbits by 2,3,7,8-tetrachlorodibenzo-p-dioxin. J. Biol. Chem. 253: 8640–8647 (1978).
24. Kitchin, K. T., and Woods, J. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) effects on hepatic microsomal cytochrome P-448-mediated enzyme activities. Toxicol. Appl. Pharmacol. 47: 537–546 (1979).
25. Madhukar, B. V. and Matsumura, F. Differences in the nature of induction of mixed function oxidase systems of the rat liver among phenobarbital, DDT, 3-methylcholanthrene and TCDD. Toxicol. Appl. Pharmacol. 61: 109–118 (1981).
26. Dees, J. H., Masters, B. S., Muller-Eberhard, U., and Johnson, E. F. Effect of TCDD and phenobarbital on the occurrence and distribution of four cytochrome P-450 isozymes in rabbit kidney, lung and liver. Cancer Res. 42: 1423–1432, (1982).
27. Okada, M., Matsui, M., Kaiu, T., and Abe, F. Studies on the glucaric acid pathway in the metabolism of d-glucuronic acid in mammals. Stimulatory effect of diphenylhydantoin and phenobarbital on the β-glucaric acid synthesis in man. Chem. Pharm. Bull. 17: 2625–2628 (1969).
28. Hunter, J., Carrarella, M., Maxwell, J. D., Stewart, D. A., and Williams, R. Urinary d-glucaric acid excretion as a test for hepatic enzyme induction in man. Lancet, 1: 572–575 (1971).
29. Maxwell, J. D., Hunter, J., Stewart, D. A., Ardenen, S., and Williams, R. Folate deficiency after anticonvulsant drugs: an investigation of hepatic enzyme induction. Brit. Med. J. 1: 297–299 (1972).
30. Hunter, J., Maxwell, J. D., Stewart, D. A., and Williams, R. Increased hepatic microsomal enzyme activity from occupational exposure to certain organochlorine pesticides. Nature 237: 399–401 (1972).
31. Williams, R., Hunter, J., and Maxwell, J. D. Measurement of hepatic enzyme induction in man. Use of d-glucaric acid excretion test. In: The Liver—Quantitative Aspects of Structure and Function, Karger, Basel, 1973, pp. 224–231.
32. Cunningham, J. L., and Price-Evans, D. A. Urinary d-glucaric acid excretion and acetylcholine pharmacokinetics before and during diphenylhydantoin administration. Eur. J. Clin. Pharmacol. 7: 89–91, 1974.
33. Latham, A. N. D-Glucaric acid as an index of hepatic enzyme induction by anticonvulsant drugs in man. J. Pharm. Pharmacol. 26: 820–826 (1974).
34. Talafant, E., Hoskova, A., and Poirier, A. Glucaric acid excretion as index of hepatic glucuronidation in neonates after phenobarbital treatment. Pediat. Res. 9: 480–483 (1975).
35. Lesamwanma, D. S. Hepatic enzyme induction and its relationship to urinary d-glucaric acid excretion in man. Brit. J. Clin. Pharmacol. 2: 546–548 (1975).
36. Latham, A. N., and Sweeney, G. D. Binding of anticonvulsant drugs to cytochrome-P-450; correlation with evidence of induction of hepatic microsomal enzymes. Can. J. Physiol. Pharmacol. 54: 844–849 (1976).
37. Latham, A. N., Turner, P., Franklin, C., and Maclay, W. Phen-
obarbitone-induced urinary excretions of D-glucaric acid and 6,β-
hydroxycortisol in man. Can. J. Physiol. Pharmacol. 54: 778–782 (1976).
38. Hunter, J., and Chasseaud, C. F. Clinical aspects of microsomal
enzyme induction. Progr. Drug Metab. 1: 129–191 (1976).
39. Ideo, G., Bellati, G., Bellobuono, A., Mocarelli, P., Mazocchi, A.,
and Brambilla, P. Increased urinary D-glucaric acid excretion by
children living in an area polluted with tetrachlorodibenzopara-
dioxin (TCDD). Clin. Chim. Acta 120: 273–283 (1982).
40. Simmonds, C. J., Davis, M., Dordoni, B., and Williams, R. Urini-
ary D-glucaric acid assay by an improved enzymatic procedure.
Clin. Chim. Acta 51: 47–51 (1974).
41. Bosnes, R. W., and Taussky, H. H. On the colorimetric deter-
mination of creatinine by the Jaffé reaction. J. Biol. Chem. 158:
581–591 (1945).
42. Introzzi, P. Trattato italiano di Medicina Interna. Tecnica e Diag-
nostica di Laboratorio, 1969, p. 253.
43. Gibbons J. J. Nonparametric Methods for Quantitative Analysis.
Holt, Rinehart and Winston, New York, 1976.
44. Dunn, O. J. Multiple comparison, using rank sums. Technometrics
6: 241–252 (1964).
45. Lehmann, E. L. Nonparametric Statistical Methods Based on
Ranks. Holden-Day, San Francisco, 1975.
46. Siegel, S. Nonparametric Statistics for the Behavioral Sciences.
McGraw-Hill, New York, 1966.