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Analysis of lipids and dioxin in chloracne due to tetrachloro-2,3,7,8-p-dibenzodioxin

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SUMMARY

Biochemical investigations on the lipid and dioxin content of the cysts and comedo-like lesions of three children exposed to tetrachloro-2,3,7,8-p-dibenzodioxin (TCDD) have been performed.

Gas chromatography and mass spectrometry failed to demonstrate the presence of dioxin despite the high sensitivity of the method. Further investigation by thin layer and gas chromatography-mass spectrometry showed that the lipids of the chloracne lesions in these children were exclusively epidermal in origin.

These findings support the hypothesis that exposure to dioxin produces a hyperproliferative reaction of the cutaneous epithelium with squamous metaplasia of the cells lining the ducts of skin glands.

Tetrachloro-2,3,7,8-p-dibenzodioxin (TCDD) is formed coincidentally during the alkaline hydrolysis of tetrachlorobenzene to produce chlorophenate, which is used as an insecticide and herbicide. Chloracne is the cutaneous response to certain chlorinated aromatic substances, of which TCDD is the prototype compound. Despite our detailed knowledge of the clinical features, and the chemical structure of the responsible compounds (Crow, 1970; Jensen, Sneddon & Walker, 1971), the pathogenesis of the disease remains obscure.

After an episode of accidental TCDD intoxication occurred at Seveso, Italy, in 1976, one of us (Gianotti, 1977) observed several children with chloracne. In this paper, the absence of dioxin from chloracne lesions and the biochemical analysis of their lipid content are reported and discussed.

METHODS

We studied three children with chloracne following contamination with TCDD: a 4-year-old boy, and two sisters aged 4 and 2 years respectively. The children were first observed a few days after the exposure to TCDD. The first histological examination was performed in the boy after 20 days, and in the two sisters after 3 months. The clinical and histological features have been reported elsewhere (Gianotti, 1977).

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Five months after the contamination, lesions were removed from all three children and their lipid content investigated. In the two sisters a further test was performed 11 months after the exposure.

Cysts and comedo-like lesions were isolated from the material obtained by curettage from face, ears and axillae. The samples were weighed (5·10 to 7·30 g in each case) and stored at −25°C until analyzed.

Dioxin determination

Two-thirds of the total material was used for dioxin determination. The material was homogenized, dehydrated in a desiccator on phosphorus pentoxide and transesterified by refluxing for 3 h with 2% (w/v) concentrated sulphuric acid in redistilled methanol (Boniforti *et al.*, 1973). Fatty acid methylesters and dioxin possibly present were extracted with anhydrous redistilled benzene; they were concentrated and then injected directly in the gas chromatographic inlet system of the mass spectrometer.

Mass spectra were obtained on the LKB Mod 9000 S combination GC-MS. The gas chromatographic inlet system was equipped with a 2 m coiled glass column, inner diameter 3 mm, packed with OV 61 3% on Chromosorb W HP 80–100 mesh. Carrier gas: helium at flow rate of 20 ml/min. Column temperature: 250°C; injector: 280°C. The molecular separator was maintained at 250°C and the ion source at 270°C.

Standard of tetrachloro-2,3,7,8-p-dibenzodioxin showed a retention time of 12 min. Mass fragmentography: TCDD focused masses: m/e 320, 322, 324. The sensitivity allowed the detection of dioxin in quantities lower than 50 pg with a signal to noise ratio higher than 4.

Lipid investigations

Lipid investigations were performed by using the technique previously applied to study the effect of ageing on fatty acids of skin surface lipids (Nazzaro-Porro *et al.*, 1979).

Extract of lipids. One third of the total material was used for lipid investigations. The bulk of the content from cysts and comedo-like lesions was obtained by extrusion and the remainder by scraping of the sac walls. During the removal efforts were made to avoid contact with subcutaneous fat. Extraction of lipids was performed with 40 ml of chloroform-methanol (2:1, v/v) for 3 h at 40°C and twice with 10 ml of chloroform-methanol (2:1, v/v) for 1 h at room temperature. Pooled extracts were dried over anhydrous sodium sulphate and lipids recovered by evaporation of solvents under reduced pressure on a rotary evaporator below 40°C.

The total lipid extracts were weighed (they represented 10–12% of the starting material weight) and then separated into their different fractions by thin layer chromatography (TLC) (Passi *et al.* 1977). The lipids on the developed chromatograms were detected by two methods: (a) charring by heating up to 150°C with 50% v/v sulphuric acid for densitometry (Nazzaro-Porro *et al.*, 1973); (b) staining with bromcresol green solution for gas liquid chromatography (GLC) (Boniforti *et al.*, 1973). The lipid spots corresponding to free fatty acids (FFA), triglycerides (TG) and sterol esters (SE) were scraped off separately from the plate and the scrapings were extracted three times with peroxide-free diethyl-ether. The recovered lipid solution was dehydrated with anhydrous sodium sulphate and evaporated nearly to dryness under a nitrogen stream.

Gas liquid chromatography

The FFA fraction was methyl esterified with diazomethane; TG and SE were separately transesterified by refluxing for 3 h with 2% (v/v) concentrated sulphuric acid in redistilled methanol. Fatty acid methylesters of SE fraction were purified from sterols by TLC (Nazzaro-Porro *et al.*, 1979). An aliquot of FA methylester mixture of each fraction was used for gas liquid chromatography on

packed column, and a capillary column was used to detect the presence of branched fatty acids. Within the branched fatty acids we were capable of identifying only the isobranched saturated (by comparison with standard reference compounds, Analabs Inc.) and isobranched unsaturated (by hydrogenation). Other positions of the methyl group in branched chains were not studied due to lack of reference standards.

Packed column. A stainless column (4 m × 2·5 mm internal diameter) containing Apiezon L as stationary phase was applied to a Dani 3900 gas chromatograph equipped with a hydrogen flame ionization detector. Helium at a flow rate of 1 ml/min was the carrier gas, splitter ratio: 1:50. The operating temperatures were: column isothermal 175°C; injector 270°C; detector 200°C.

Double bond position of unsaturated fatty acids.

(1) Performic acid peroxidation of fatty acid methylesters: the remaining fatty acid methylester mixtures of each of the three fractions (triglycerides, free fatty acids and sterol esters) were weighed before conversion to diols or polyhydroxymethylesters. Each mg of the samples was allowed to react with 15 μl of formic acid and 2 μl of 30% hydrogen peroxide at 40°C for 2 h with occasional shaking. The reaction mixture was diluted with 1 ml distilled water, extracted three times with 5 ml portions of peroxide-free redistilled diethyl-ether, the extract pooled and the ether evaporated to dryness under nitrogen stream. The product was saponified in 0·1 ml 3N sodium hydroxide at 100°C for about 30 min, acidified with diluted sulphuric acid and extracted three times with 5 ml portions of peroxide-free diethyl-ether. The pooled extracts, containing saturated dihydroxy and polyhydroxy fatty acids, were evaporated to dryness under a nitrogen stream.

(2) Formation of trimethylsilyloxy derivatives: The previous mixtures were methylated with diazomethane. The corresponding dihydroxy fatty acid methylesters were converted to the respective trimethylsilyloxy derivatives (TMSO) by treatment with 0·2 ml of N,O-bis-(trimethylsily)-acetamide (BSA) and trimethylchlorsilane (TMCS) (5:1) in pyridine. The mixtures were allowed to stand for 5 min and injected directly in the gas chromatographic inlet system of the mass spectrometer. Mass spectra were obtained on LKB Mod 9000 S combination GC-MS. The gas chromatographic inlet system was equipped with a 3 m coiled glass column packed with 5% OV-101 in Chromosorb W HP 80–100 mesh. Carrier gas: helium at flow rate of 25 ml/min. Column temperature was initially isothermal at 180°C for 10 min and then the column temperature was programmed from 180°C to 230°C at the rate of 3°C/min and isothermally thereafter. The He separator was maintained at 250°C and the ion source at 270°C. Ionizing potential was 20 eV and the ionizing current was 60 mA. Average spectra from respective scanning were recorded for each gas chromatographic peak.

Gas liquid chromatography of sterols. The dry sterol extracts were converted to the corresponding TMSO derivatives (trimethylsilyloxy) by treatment with 0·5 ml of a pyridine-hexamethyldisilazone (HMDS)-trimethylchlorsilane (TMCS) (103:3:1). The mixture was allowed to stand for 5 min and injected directly in a Carlo Erba Mod.GV equipped with a hydrogen flame ionization detector. A 3 m coiled glass column packed with 3% OV 17 on silanized acid-washed Chromosorb W HP 80–100 mesh, was applied to the instrument. Flow rate was 30 ml/min of nitrogen carrier gas. The operation temperatures were: column isothermal 250°C; injector 280°C; detector 230°C.

RESULTS

Dioxin determination

No dioxin was detected by the GC-MS analysis of lipid material.

Lipid investigation

Table I shows the different classes of lipids obtained by TLC. It is evident that wax esters and squalene were absent, while sterols and sterol esters were very relevant, representing 65% of total lipids. The gas chromatographic analysis of sterols and sterol esters showed that the main sterol component, in both the fractions, was cholesterol (99%), lathosterol being present only in traces. The free fatty acid fraction was well represented, while triglycerides were in low proportion. There was no significant difference in the lipid composition of cysts and comedo-like lesions analysed at different times after exposure to TCDD.

TABLE 1. Percentage lipid composition of chloracne lesions in children exposed to TCDD

Cases	Time from exposure	DG (1–2, 2–3, 1–3)	ST	FFA	TG	WE	SE	SQ
1.	5 months	1·8	44·2	22·2	7·9	—	23·8	tr
2.	5 months	3·3	36·8	25·7	9·8	—	24·3	tr
	11 months	3·6	44·7	21·3	8·9	—	21·4	tr
3.	5 months	1·8	43·4	17·7	9·3	—	27·7	tr
	11 months	3·0	46·9	18·8	11·6	—	19·6	tr

DG, diglycerides; ST, sterols; FFA, free fatty acids; TG, triglycerides; WE, wax esters; SE, sterol esters; SQ, squalene.

Phospholipids were not considered; because of their polarity they remain at the origin.

Analytical data on fatty acid composition and double bond position of the unsaturated fatty acids of free fatty acids, triglycerides and sterol esters classes of lipids of chloracne of the three children are not reported here in detail; the relative amounts of the different fatty acid types of free fatty acids, triglycerides and sterol esters are reported in Table 2. In all the three classes of lipids we can observe

TABLE 2. Relative amounts of the different fatty acid types of FFA, TG and SE classes in chloracne lipids and their Δ6 and Δ9 patterns

Lipid classes	Straight fatty acids mean%					Branched fatty acids mean %			Others§ mean%
	Saturated	Monounsaturated		Polyunsaturated			Unsaturated		
		Δ6	Δ9	Δ6	Δ9	Saturated	Δ6	Δ9	
Free fatty acids	32·99	tr	26·58	tr	26·65*	tr	—	—	13·68
Triglycerides	35·12	tr	27·34	—	18·73†	tr	—	—	18·71
Sterol esters	24·66	tr	27·66	—	24·44‡	tr			21·14

Relative amounts of the different fatty acid types were calculated by the results obtained by capillary and packed column. The structure of the unsaturated fatty acids was studied by GLC-MS analysis of TMSO derivatives of fatty acid methylesters (for details see text).

tr = < 0.1%.

* C18:2, 13·76%; C18:3, 2·48%; C20:2, 1·77%; C20:4, 4·55%.

† C18:2, 12·30%; C18:3, 3·45%; C20:2, 0·65%; C20:4, 2·35%.

‡ C18:2, 14·77%; C18:3, 4·12%; C20:2, tr; C20:4, 9·15%.

§ Others were constituted by fatty acids with chain length over C20:4 which have not been identified due to lack of standards.

that: (a) the branched fatty acids, either saturated or unsaturated, were present only in traces (<0·1%) or absent, (b) within the straight fatty acids a very high percentage was represented by polyunsaturated fatty acids—the 'others' were constituted by fatty acids with chain length over C20:4 which have been calculated but not identified due to lack of standards; (c) all the unsaturated fatty acids, from the lowest to the longest chains, had a Δ 9 pattern, and C18: 2 was exclusively represented by C18:9–12, i.e. linoleic acid.

DISCUSSION

The GC-MS analysis of lipids extracted from the pooled material obtained by curettage of chloracne lesions in children exposed to TCDD failed to demonstrate the presence of dioxin. The fact that dioxin was not detected despite the high sensitivity of the method (the weight of starting material from each case varied from 3·40 to 4·86 g and the method allowed the detection of dioxin in quantities lower than 50 pg) might be related to the time elapsed between the contamination and the investigation. If the substance is still present, it does not appear to be excreted in skin lipids.

The lipid investigations were performed in order to determine whether the lipids were of sebaceous and/or epidermal origin. As reported by Crow (1970), the clinical and experimental data in adult humans and in animals suggest that comedones and cysts of chloracne are due to a keratinizing metaplasia of the epithelial lining of the pilosebaceous follicles associated with precocious atrophy of sebaceous glands (Shelley & Kligman, 1957); in the experimental rabbit it has been shown that sebaceous gland atrophy even precedes the development of an occluded comedo (Hambrick, 1957). Other authors (Cunliffe *et al.*, 1975) suggest that chlorinated hydrocarbons induce chloracne by modifying the metabolism of the pilosebaceous follicle.

The lipids of epidermal origin differ greatly from those derived from sebaceous glands. Most studies in this field have been carried out by analysing skin surface lipids which are derived mainly from the sebum with a small proportion from the Malpighian layer of the epidermis. The relative contribution of lipids from each of these sources depends upon the degree of activity of sebaceous glands which, being under hormonal, and especially androgenic control (Ebling, 1974; Pochi & Strauss, 1974), varies with age and sex. Prepubertal children and old people have a low sebaceous activity as compared with young or adult subjects; nevertheless sebaceous lipids are always present in their skin surface lipids indicating a continuous activity of sebaceous glands (Cotteril *et al.*, 1972; Nazzaro-Porro *et al.*, 1979).

In addition to the studies on skin surface lipids at different ages and on cutaneous areas more or less rich in sebaceous glands (Greene *et al.*, 1970), investigations on whole isolated sebaceous glands (Kellum, 1967) and on isolated epidermis (Ansari, Nicolaides & Fu, 1970) have been performed. These studies have shown that the lipid classes characteristic of sebum are wax esters and squalene; that sterols are in high prevalence in lipid of epidermal origin and that they are represented mainly by cholesterol, that sterol esters are formed by esterification of cholesterol with fatty acids coming both from epidermis and sebaceous glands (Freinkel & Aso, 1969; Nicolaides, 1963) and that triglycerides and free fatty acids are of both sebaceous and epidermal origin.

The researches on fatty acid composition of the different lipid classes (Nicolaides & Wells, 1957; Downing & Strauss, 1974; Nikkari, 1974; Nazzaro-Porro *et al.*, 1979) have shown that epidermis and sebaceous glands can make the same kind of fatty acids but in widely different amount. Sebum has a significant amount of fatty acids not commonly found in nature as odd chain length fatty acids or branched fatty acids; in addition, the level of unsaturation of the unsaturated fatty acids of sebum characteristically presents a Δ6 pattern, which is very rare in nature. Epidermis produces less odd and branched fatty acids than sebum and, as in most living tissues, most of the unsaturated fatty

acids have a Δ9 pattern. Within the polyunsaturated fatty acids present on the skin surface, the most abundant is represented by C18:2, the others being present only in a very low proportion. C18:2 presents two isomers: C18:9–12, the essential fatty acid 'linoleic acid' and C18:5–i, characteristic of sebaceous lipids, named in fact 'sebaleic acid' (Nicolaides, 1974).

In our investigations, the TLC analysis of lipid in cysts and comedo-like lesions of children with chloracne showed the complete absence of the characteristic sebaceous lipid (wax esters and squalene), and a high content of epidermal lipid (sterols and sterol esters), the main sterol being cholesterol. These findings are similar to those reported in a study of lipid content of keratinous cysts of epidermis (Nicolaides, 1968) but different from those obtained from comedones of acne vulgaris in which sebaceous lipids are always present (Nicolaides *et al.*, 1970; Nazzaro-Porro *et al.*, 1973).

From Table 1 it is also evident that free fatty acids occur in high percentage while the triglycerides are at a low level. In skin surface lipids and in comedones from acne vulgaris, the free fatty acids are mainly generated by the hydrolysis of triglycerides due to the lipolytic activity of cutaneous skin flora (Kellum, 1967; Nicolaides & Wells, 1957; Nicolaides, 1973). Recent experiments, however, indicate that during epidermal keratinization a process of rescue of lipids liberated by the degenerating corneum cell membranes takes place, the neutral lipids becoming esterified with fatty acids derived from hydrolysis of phospholipids (Freinkel, 1979). That a similar mechanism occurs in the genesis of free fatty acids in chloracne is suggested by the strong similarity in the fatty acid composition of the three fractions, triglycerides, free fatty acids and sterol esters (Table 2). The epidermal origin of all three lipid fractions is revealed by the gas chromatographic analysis showing the absence, or the presence only in traces, of branched fatty acids and the very high percentage of polyunsaturated fatty acids, many of which have a chain length over C18:2. It is further confirmed by the study of the double bond position of the unsaturated fatty acids showing that all the unsaturated fatty acids, from the shortest to the longest chains, have a Δ9 pattern, and that the more representative C18:2 is exclusively formed by C18:9–12, linoleic acid (Table 2).

The analysis of the composition and structure of lipids of chloracne in our children gave similar results in all the investigations performed in spite of the different features of the cutaneous lesions. These varied, according to the time elapsed from the contamination with TCDD, from severe and diffuse hyperkeratotic lesions with granulomatosis, to cystic masses and follicular comedo-like hyperkeratosis (Gianotti, 1977). The biochemical data, demonstrating the epidermal origin of lipid content of cutaneous lesions, were in line with the histological findings, which showed the presence of epidermal hyperplasia with hyperkeratosis and a squamous metaplasia of the cells lining the duct of the sweat glands (Gianotti, 1977). According to the data reported in adult chloracne, in which the squamous metaplasia affects mainly the cells of the sebaceous gland duct, the cutaneous pathology induced by exposure to dioxin seems to be related to a hyperproliferative reaction of the entire cutaneous epithelium. Whether the squamous metaplasia is a temporary condition, due to the direct toxic effect of dioxin, will be clarified only after long term observation.

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