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IDENTIFICATION OF DIETHYLENE GLYCOL IN SERA FROM EGYPTIAN CHILDREN BY FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

Sera taken from fifteen patients (from Kerdasa village near Cairo, Egypt) infected with *Schistosoma haematobium*, with eggs present in the urine, were studied by frequency-pulsed electron-capture gas—liquid chromatography (FPEC—GLC). Some of the patients were treated with metrifonate and again studied by FPEC—GLC. Diethylene glycol was detected in the sera of untreated patients infected with *S. haematobium*. This compound was identified by negative chemical ionization and electron-impact mass spectrometry. Initially we suspected that the build-up of diethylene glycol in these patients was caused by schistosomiasis infection. However, in a follow-up blind-coded study using FPEC—GLC, which included 37 sera from Kerdasa and Tamooh villages near Cairo, Egypt, we detected diethylene glycol in eleven samples, four of which were controls from the villages. These latter findings indicate that the source of diethylene glycol might be the environment or foodstuffs, but the specific source has not been determined. Regardless of the source, diethylene glycol could affect the health of these Egyptian children by causing a narcotic effect, increased bladder stones, and increased numbers of bladder tumours.

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INTRODUCTION

While conducting studies of sera from *Schistosoma*-infected children in Kerdasa, a village near Cairo, Egypt, we detected an unknown peak by frequency-pulsed electron-capture gas—liquid chromatography (FPEC—GLC). We thought this unknown peak might be associated with schistosomiasis. Schistosomiasis is a disease that is endemic and chronic in many areas of the world. Little is known about changes that take place in body metabolism or what products are produced in vivo by these organisms [1], but it is known that patients infected with *Schistosoma haematobium* have a higher incidence of bladder cancer, and that the disease seems to produce a narcotic effect on the host [1]. We decided to try and identify this unknown peak by gas chromatography—mass spectrometry (GC—MS).

FPEC—GLC is a selective sensitive analytical tool that has been used to detect changes in spent culture media [2—5] and from body fluids of diseased persons [5—10]. Mass spectrometry (MS) has been commonly used in combination with gas chromatography (GC) to identify unknown peaks detected by this technique. Identification by GC—MS of unknown peaks detected by FPEC—GLC analysis of body fluids is often hard to accomplish. The reasons for the difficulty lie in the fact that: FPEC—GLC often surpasses the detection limits of GC—MS, or that there is failure to detect a molecular ion in electron-impact spectra of fluorinated derivatives. The purposes of this study were as follows: (1) to find GC—MS conditions capable of obtaining electron-impact and chemical ionization spectra (both positive and negative) of the unidentified peak detected in the FPEC—GLC analysis of serum; (2) to identify the unknown compound, if possible; (3) to confirm identification by derivatization and analysis of the suspected compound by FPEC—GLC and GC—MS; and (4) to search the literature for information concerning the toxicity, physiological effects, and possible biosynthesis of the compound.

MATERIALS AND METHODS*

Serum specimens

The 1 ml of serum used for FPEC—GLC analysis in the study was serum left after routine tests had been performed. The first samples were collected from the village of Kerdasa. The control specimens were collected from employees at the Biomedical Research Center in Cairo. The samples were stored at −20°C until they were used. The median age, sex, and treatment regimen of the patients, and number of specimens used in the first study are given in Table I. The Biomedical Research Center for Infectious Diseases (BRCID) in Cairo, Egypt, confirmed *S. haematobium* infection by egg count in the urine. Following the first study, a second blind-coded study was conducted which involved FPEC—GLC analysis of sera from 37 additional children. The samples for the second study were taken from two villages near Cairo, those of Kerdasa and Tamooh. The samples consisted of fourteen control sera from the villages, nineteen sera

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

SUMMARY OF INFORMATION RELATING TO SPECIMENS USED IN THE FIRST FPEC—GLC STUDY

	No. of cases studied	Age range (years) (median)	Male:female	Egg count* (E.C.) high:mod.:low	No. of patients treated** (E C. before treatment: E.C. after treatment)
S. haematobium	15	7—12 (11)	11:4	8:3.4	5 (1230:25), (500:32), (220:100), (188:15), (50:1)
Control	5	19—55 (27)	4:1	N/A	N/A

*For *S. haematobium*, high E.C. > 400 per 10 ml of urine, moderate E.C. = 100—400 per 10 ml of urine, low E.C. <100 per 10 ml of urine.
**Sample taken from patient after two weeks of treatment with metrifonate (one course, 7.5 mg/kg)

from patients infected with *S. haematobium* and four sera from patients infected with *S. mansoni*. *S. mansoni* infection was confirmed by faecal egg count at the BRCID.

Extraction and derivatization procedures

Each serum specimen was placed in a 50-ml round-bottomed centrifuge tube with a Teflon-lined screw cap; then heptanoic acid (3.15 μmol in 0.1 ml distilled water, made basic to about pH 10 with sodium hydroxide to obtain solubility) and di-*n*-butylamine (1.19 μmol in 0.4 ml of distilled water made acidic to about pH 2 to increase solubility) were added to each sample as internal standards. Next, the samples were acidified (about pH 2) with 0.1 ml of 50% (v/v) sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell Wrist Action shaker at a setting of 10 to obtain carboxylic acids and alcohols. The residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *M* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction, to obtain the amines. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carboxylic acids and HFBA esters of alcohol as described previously [2, 4]. The basic chloroform extracts containing amines and alcohols were derivatized with HFBA pyridine—ethanol to form amides as described previously [11]. A few HFBA derivatives of alcohols were prepared from pH 2 chloroform extracts using the procedure described [3]. After derivatization the samples were dissolved in 0.1 ml of xylene—ethanol (1:1). A 2-μl injection was used for analysis. The techniques for filling and cleaning the syringe have been described [4].

Apparatus

The derivatives were analysed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10 mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80—100 mesh Chromosorb W (HP AW DMCS) were used under conditions previously described [2, 10].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS-16 revision B) accumulated data from the gas chromatograph, analysed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid and di-*n*-butylamine, which were added as internal standards [2].

FPEC—GLC identification of the unknown peak 7 was made by comparing a known standard on an OV-101 packed column and on a 50-m OV-101 fused-silica capillary column (Perkin-Elmer) programmed as follows: isothermal at 100°C for 12 min, then 60°C/min to 265°C. We used a capillary column with a splitless injector. A 30-sec solvent vent was used, and helium was the carrier gas at a flow-rate of 3 ml/min. The recorder chart speed was 2.54 cm/min.

GC—MS analysis

A Finnigan 4023 gas chromatograph—mass spectrometer—data system (GC—MS—DS) equipped with a 25-m fused-silica column coated with OV-101 (Hewlett-Packard) was used. The capillary column was connected directly to the inlet of the MS source using a guide tube. The mass spectrometer was fitted with the pulsed positive ion/negative ion chemical ionization (PPINICI) accessory, allowing both positive and negative ion detection. The mass spectrometer was operated at an electron multiplier setting of 1200 V; the ionizing voltage was 70 V for all studies. We used helium as the carrier gas and methane (Matheson) as the reagent gas for chemical ionization with a source pressure at $1.2 \cdot 10^{-5}$ torr.

RESULTS

In the first study a compound was consistently detected by FPEC—GLC in the sera of fifteen untreated patients infected with *S. haematobium*, but this compound was not detected in the normal controls. As can be seen (Fig. 1A—C, peak 4) the compound was extractable under either acidic or basic conditions. It did not react with TCE, but did react with HFBA to form an electron-capturing derivative. The peak was found to be greatly reduced or eliminated after two weeks of effective therapy in five patients (Fig. 2A—C) and was not detected in the initial set of control specimens (Fig. 2D) which were obtained from laboratory personnel. The behaviour of the compound upon extraction and derivatization suggested that it contained hydroxyl group(s). The combined serum extracts from four different patients were necessary to obtain the electron-impact (EI) mass spectrum shown in Fig. 3. No molecular ion was observed for the derivative, but the observation of a fragment ion at *m/z* 213 is consistent with the presence of hydroxyl groups in the original compound. The other suggested structures for ions observed in the spectrum are those typically observed for fluorinated compounds [12]. No additional molecular weight information was obtained through positive chemical ionization. The negative chemical ionization (NCI), which is especially sensitive to halogens, was more sensitive than EI ionization by a factor of 5—10. The fluorinated derivatives react under NCI conditions through a dissociative resonance capture mechanism. Such a mechanism results in observed fragment ions $(M-20)^-$ and neutral HF. The fragment ion *m/z* 478 (Fig. 4) thus suggests a molecular weight of 498. The requirements of a 498 molecular weight and the hydroxyethylene structural unit suggested by *m/z* 241 in the EI spectrum limit the number of possible chemical structures. The analysis of the fluorinated derivative of diethylene glycol by co-chromatography on capillary and packed columns yielded identical retention times and mass spectra in EI and NCI to the un-

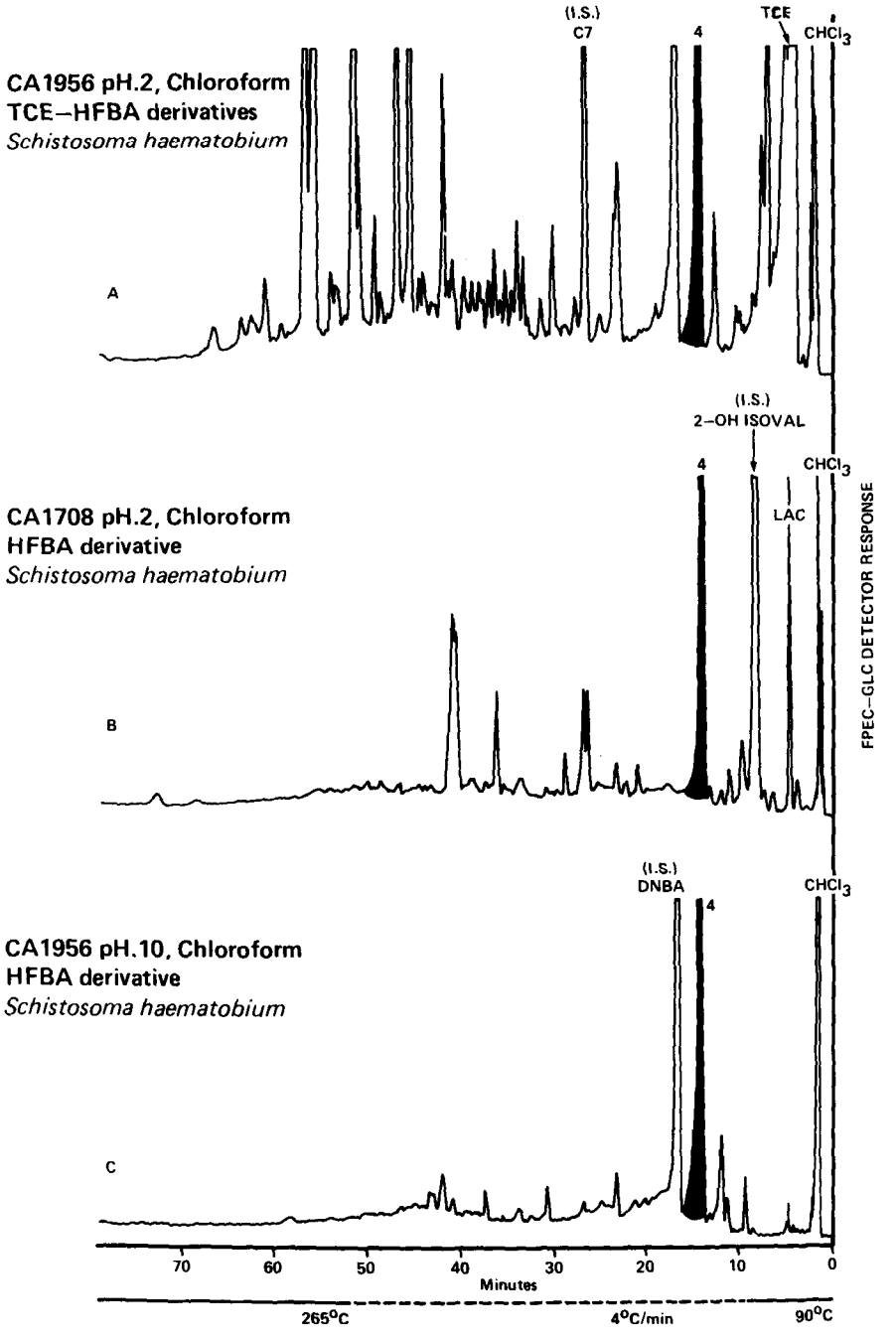

Fig. 1. FPEC—GLC chromatograms from serum analysed on OV-101 packed columns. The type of disease, extraction procedures, and type of extraction are indicated. TCE = trichloroethanol, HFBA = heptafluorobutyric anhydride, I.S. = internal standard, $CHCl_3$ = residual chloroform. The letter "C" before a number indicates a saturated straight-chain carboxylic acid with the number of the carbon atoms indicated by the number. The letter "i" indicates "iso" and the use of colon between two numbers indicates unsaturation. Peak 4 has been determined to be diethylene glycol. The samples were from Kerdasa village near Cairo, Egypt.

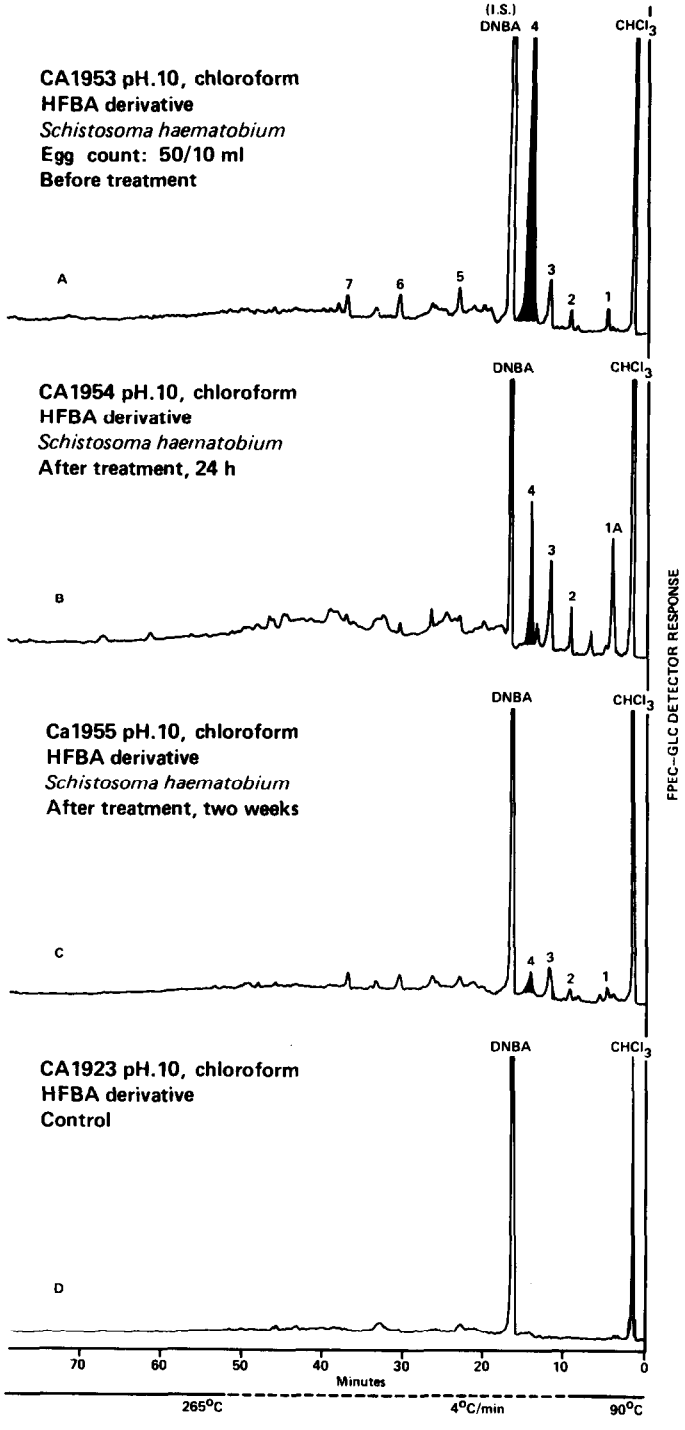

Fig. 2. FPEC—GLC chromatograms from sera analysed on OV-101 packed columns. The type of extraction and derivative are indicated. I.S. = internal standard, DNBA = di-*n*-butylamine, $CHCl_3$ = residual chloroform; a number or letter over a peak is an unidentified peak. The control serum was taken from laboratory personnel. Note that some diethylene glycol (peak 4) was carried over into the basic extraction.

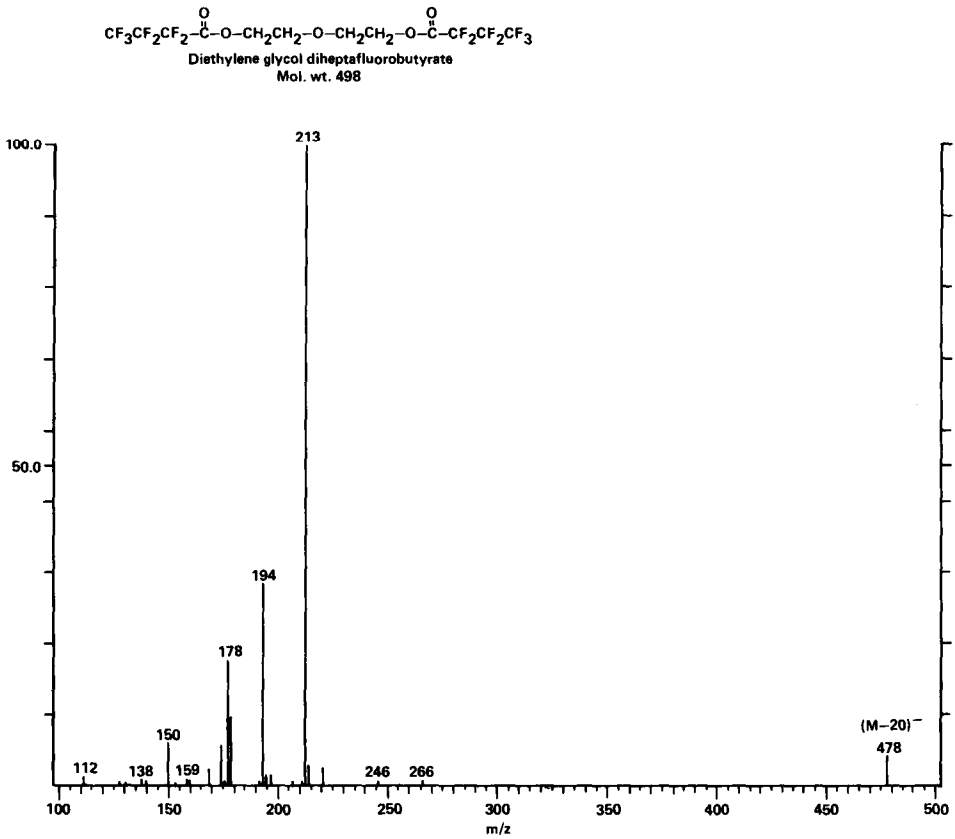

Fig. 3. Electron-impact mass spectrum of peak 4 (Fig. 1) obtained from an OV-101 fused-silica capillary column. Note that the spectrum is identical to the spectrum of authentic diethylene glycol diheptafluorobutyrate. Conditions are described in the text.

known gas chromatographic peak 4 (Fig. 1) and the unknown compound was identified as the HFBA diester of diethylene glycol.

In the second, blind-coded study diethylene glycol was detected by FPEC—GLC in about one fourth of the quantity detected in the first study, and then in only eleven of the 37 sera tested. Four of the samples containing diethylene glycol were controls, one was from a patient positive for *S. mansoni*, and the other six were from sera of patients infected with *S. haematobium*.

DISCUSSION

Our initial study results suggested the possibility that diethylene glycol might be a metabolic marker characteristic of infection with *S. haematobium*. The amounts present were about 11 nmol/ml of serum, a quantity that would require the ingestion of far greater amounts if the source was environmental or foodstuffs rather than that produced in the course of the infection. However, in our follow-up study in which 37 sera from Kerdasa and Tamooh were analysed, diethylene glycol was detected in moderate-to-trace amounts in eleven sera, four of which were from normal individuals and one of which was from a

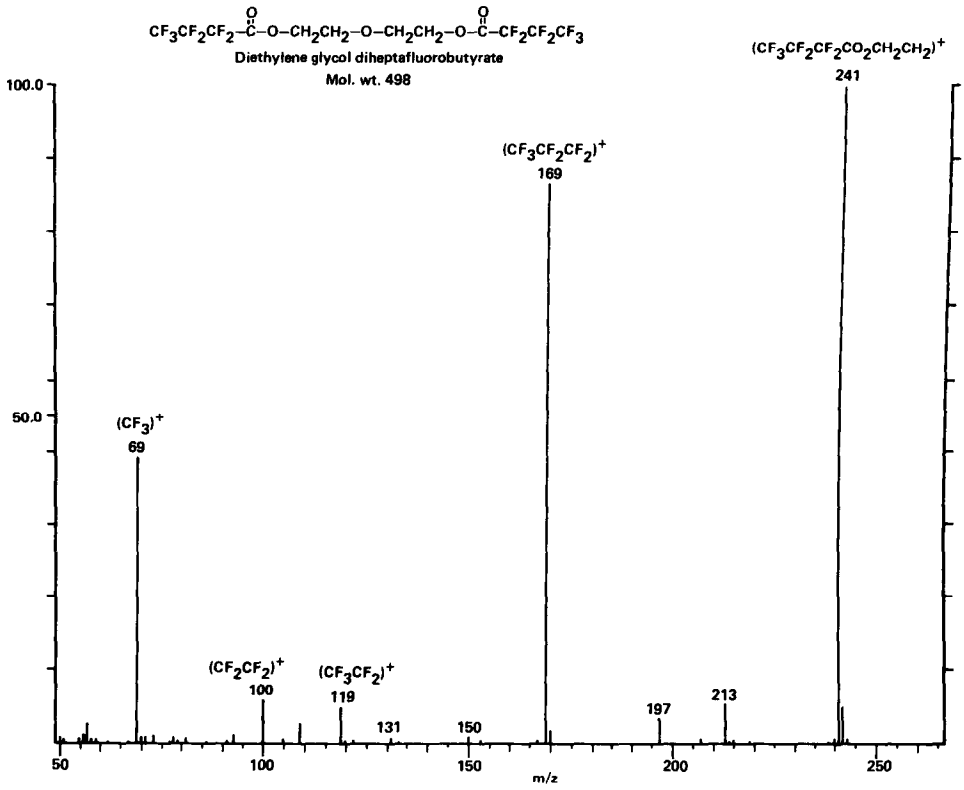

Fig. 4. Negative chemical ionization spectrum of peak 4 (Fig. 1) obtained from an OV-101 fused-silica capillary column. Note that the spectrum is identical to the spectrum of authentic diethylene glycol diheptafluorobutyrate.

person found to have a high *S. mansoni* egg count. We do not know of an environmental or food source by which these children could have been exposed to diethylene glycol. The median age of the patients was eleven years, which makes industrial exposure unlikely. Furthermore, serum levels of the compound decreased in amount in five patients following effective anti-schistosomal therapy, an observation which seems to indicate some relationship to the disease process. When patients did not respond to therapy, as indicated after two weeks by a consistant egg count, diethylene glycol was still present.

A computer search of the literature on biosynthesis, fermentation synthesis, and toxicity of diethylene glycol revealed that a great deal of research had been done on the toxicity of this compound, but we found no reports on its biosynthesis. Diethylene glycol is a central nervous system depressant, and a single lethal oral dose for man is approximately 1 ml/kg [13]. Based on a study [13] of 105 fatalities among 353 people who ingested diethylene glycol that contaminated a solution of sulphanilamide, symptoms included nausea, dizziness, and pain in the kidney region. Death resulted from renal failure [13]. A study conducted by Fitzhugh and Nelson [14] on rats showed that, at a 4% dietary level, there was depression of growth, formation of bladder stones, severe kidney damage, moderate liver damage, and frequent appearance of bladder tumours. Woo et al. [15] studied the volatile compounds present in the urine

of rats administered *p*-dioxane, an hepatic carcinogen in this species, and reported that the metabolite *p*-dioxan-2-one was detected. They also detected the same metabolite when diethylene glycol was administered to the rats.

Published data on the physiological effects of diethylene glycol showed similarities to published data on patients chronically infected with *S. haematobium*. The major correlations were that patients infected with *S. haematobium* experienced a narcotic effect, developed bladder stones, and showed increased incidence of bladder cancer [1].

In view of the facts that our second blind-coded study, involving two villages, revealed that diethylene glycol was found in some of the controls, was missing in some of the patients with schistosomiasis, and was detected in the serum of a patient infected with *S. mansoni*, it is likely that this compound is an industrial product that has become an environmental or food contaminant. Although no proof exists, clothing fabric treated with diethylene glycol or possibly corks in soft drink bottles could be a source of the compound. It has been two years since the study was completed and no known source has yet been identified. Whatever the specific source, the possibility exists that diethylene glycol may have an effect on the health of some exposed Egyptian children.

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