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Evaluation of the genetic and embryotoxic effects of bis(tri-*n*-butyltin)oxide (TBTO), a broad-spectrum pesticide, in multiple in vivo and in vitro short-term tests

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Summary

The genetic and embryotoxic effects of bis(tri-*n*-butyltin)oxide (TBTO) were evaluated in multiple in vivo and in vitro short-term tests preparatory to its potential wide use as a molluscicide in control of schistosomiasis. When tested in the *rec* assay in *Bacillus subtilis*, TBTO was not mutagenic and it did not induce reverse mutations in *Klebsiella pneumoniae*. Neither in the presence nor in the absence of rat liver activation system did TBTO produce point mutations in *Salmonella typhimurium* strains TA1530, TA1535, TA1538, TA97, TA98 or TA100. TBTO was mutagenic in strain TA100 in a fluctuation test, but only in the presence of rat liver S9 (Aroclor-induced).

TBTO did not induce gene mutations in the yeast *Schizosaccharomyces pombe*, mitotic gene conversions in the yeast *Saccharomyces cerevisiae*, nor sister-chromatid exchange in Chinese hamster ovary cells in the presence or absence of rat or mouse liver S9. In the latter cells, structural chromosomal aberrations, endoreduplicated and polyploid cells were induced.

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Abbreviations: TBTO, bis(tri-*n*-butyltin)oxide; S9, Aroclor-induced rat liver 9000 × *g* fraction; DMSO, dimethyl sulphoxide; SCE, sister-chromatid exchange; CHO, Chinese hamster ovary; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte.

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TBTO did not induce gene mutations in V79 Chinese hamster cells (to 8-azaguanine-, ouabain- or 6-thioguanine-resistance) in the presence of a rat liver postmitochondrial fraction or in cell (hamster embryo cells and human and mouse epidermal keratinocyte)-mediated assays. In mouse lymphoma cells, TBTO did not induce 6-thioguanine- or BUdR-resistant mutations.

As many tumour promoters inhibit metabolic cooperation between V79 Chinese hamster 6-thioguanine-resistant/-sensitive cells, TBTO was tested but showed no such activity.

TBTO was examined for the induction of recessive lethal mutations in adult Berlin K male *Drosophila melanogaster,* either by feeding or by injection. Doses of 0.37 or 0.74 mM did not increase the number of X-linked recessive lethal mutations.

An increased number of micronuclei was observed in the polychromatic erythrocytes of male BALB/c mice 48 h after a single oral dose of TBTO (60 mg/kg bw), while a lower dose (30 mg/kg bw) was ineffective. Neither of the two doses had induced micronuclei 30 h after treatment.

The reproductive toxicity of TBTO was studied in NMRI mice. In a 10-day toxicity study, the LD_{50} and LD_{10} were 74 and 34 mg/kg bw, respectively. An increased frequency of cleft palates was seen in the fetuses of mice (compared with controls, 0.7%) treated orally during pregnancy with 11.7 mg/kg TBTO (7%), 23.4 mg/kg (24%) or 35 mg/kg (48%). The two highest doses of the drug also increased the frequencies of irregular ossification centres of sternebrae and of minor abnormalities, such as fusion of the bases of the os occipitalis. Doses of 6 mg of TBTO/kg bw do not produce teratogenic effects in mice. Teratogenic effects were only seen at TBTO doses that were toxic to the maternal organism. Electron microscopy 26 and 48 h after TBTO treatment showed no evidence that embryos were damaged. In contrast, the maternal liver was greatly affected.

The tin contents of different organs of the maternal organism and of the whole fetus were determined after TBTO treatment. After a single oral treatment with 117 mg TBTO/kg bw, the total tin content in 11.5-day-old embryos 12 h after TBTO administration was 9.5 nmoles Sn/g wet weight — 70% greater than the normal value. The total concentration of tin in the maternal liver was increased to a similar extent (by approximately 60%) over the normal level between 6 and 24 h after treatment. No other organ studied showed similarly high increases in tin content.

These results show that TBTO gives negative results in short-term tests using various genetic endpoints. However, at cytotoxic concentrations, it was mutagenic in one bacterial strain, clastogenic in CHO cells in vitro and produced micronuclei in mouse bone marrow cells in vivo. In view of these adverse biological effects, and its high capacity to interfere with morphogenetic differentiation processes in vitro careful evaluations must be made before TBTO is released in large quantities into the aquatic environment, and levels to which humans are exposed must be strictly controlled when it is used as a molluscicide.

Schistosomiasis (bilharziasis) is essentially a man-made parasitic infection, affecting more than 200 million people in 74 endemic countries (WHO, 1985). Several species of schistosomes exist; these are acquired by man through contact with infected fresh water. The parasites rapidly penetrate the skin and migrate and develop to maturity in the blood vessels of the intestines (intestinal schistosomiasis: *Schistosoma mansoni; S. japonicum*) or of the urinary bladder (urinary schistosomiasis: *S. haematobium*). When the eggs laid by the fertilized female worm reach fresh water, an immature form of the parasite (miracidium) hatches and may then enter a particular species of snail which is specific for each species of schistosome, in which it is further transformed. After full development in the snail, new larval infective forms of the parasite (cercariae) escape; these can again rapidly penetrate human skin.

Control of schistosomiasis is more feasible than ever before due to the availability of safe and effective antischistosomal drugs, low-cost diagnostic techniques as well as important advances in our understanding of the epidemiology of this disease.

In contrast, means for controlling the snail intermediate hosts have not improved markedly, and the impetus to develop them may have re-

gressed in recent years. At present, only one chemical molluscicide — niclosamide — is used in most control programmes. The relatively high costs of obtaining and applying this compound limit its use to those countries where there is sufficient financial and organizational capacity and which place high priority on the control of schistosomiasis (McCullough and Mott, 1983).

The development of new synthetic molluscicides to augment the impact of the other advances in schistosomiasis control is badly needed. It has long been known that a number of organotin compounds have high molluscicidal activity essentially in trisubstituted tin molecules. In particular bis(tri-*n*-butyltin)oxide (TBTO) could be a very effective molluscicide in certain sites and in controlled-release formulations. It cannot be used in the field, however, until definitive long- and short-term studies of its toxicity have been completed.

Since no such data existed in the published literature, we implemented a multi-centre trial to evaluate the genetic and embryotoxic effects of TBTO in multiple in vitro and in vivo short-term tests, as was done previously for the antischistosomal drug praziquantel (Bartsch et al., 1978). The results for TBTO are reported below.

Materials and methods

Chemicals. The broad-spectrum pesticide TBTO (CAS Name, distannoxane, hexa-*n*-butyl-; CAS Reg. No., 56-35-9; MW 596.1; molecular formula, $C_{24}H_{54}OSn_2$, density, d_4^{20}, 1.17) was a product of M and T Chemicals Inc., Vlissingen, The Netherlands (purity, 95.3%; Lot No. CH 356). This batch was used for all the studies reported herein. All other chemicals were obtained commercially.

Cotton-seed oil, pluronic F-68 and lecithin for injection experiments in *Drosophila melanogaster* were kindly provided by Dr. P.E. Schurr, Upjohn Company, Kalamazoo, MI, U.S.A.

(1) DNA-damaging and mutagenic activity in Bacillus subtilis, Salmonella typhimurium and Klebsiella pneumoniae

(1) Test organisms. *B. subtilis* strains H17 rec$^+$ and M45 rec$^-$ were used for the rec assay according to Kada et al. (1980). *S. typhimurium* TA100, TA98, TA97, TA1535, TA1538 and TA1530 were provided by B.N. Ames, Berkeley, CA, U.S.A. Cultures were grown overnight from frozen stocks (−70°C) in Difco nutrient broth (plate assay) or in Oxoid medium No. 2 fortified with 10 μg/ml L-histidine to avoid any selection artefacts (fluctuation test). The presence of R-factor in TA100, TA98 and TA97 was checked by seeding bacteria on agar that contained ampicillin. The cultures were checked for crystal violet sensitivity and mutability using methyl methanesulphonate and benzo[*a*]pyrene in the presence of a metabolic activation system for TA100, 4-nitroquinoline *N*-oxide for TA97 and TA98, 2-nitrofluorene for TA1538 and *N*-nitroso-*N'*-methyl-*N'*-nitroguanidine for TA1530 and TA1535. *K. pneumoniae* ur$^-$ pro$^-$ strains were used in a fluctuation test (Voogd et al., 1977) without rat liver postmitochondrial fraction (S9).

(2) Preparation of rat liver postmitochondrial fraction (S9). Adult BD VI rats (~ 120 g) bred in the IARC laboratory, were fed on a Charles River CRF diet. Groups of 4–6 rats received an intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) 5 days prior to sacrifice. A postmitochondrial fraction (9000 × *g*) from pooled rat livers was prepared and stored at −80°C, as described previously (Ames et al., 1975; Malaveille et al., 1982).

In another series of experiments (Table 1, Expt. No. 3), S9 was prepared from male, Aroclor-pretreated Sprague–Dawley rats (250 g, from Centraal Proefdieren Bedrijf TNO, Zeist, The Netherlands) that were given Hope Farms pellets and water ad libitum. The protein content of the S9 was 36 mg/ml. To prepare 10 ml S9 mix, 3 ml S9 liver fraction were used. Each selection plate contained 20 ml selection medium with 3 ml top agar (2.7 ml soft agar, 0.1 ml bacteria, 0.1 ml solution of test compound in dimethyl sulphoxide and 0.1 ml S9 mix or 0.85% saline), as described by Ames et al. (1975).

(3) Isolation of rat hepatocytes. Adult male BD VI rats were used to isolate 90% viable hepatocytes (trypan blue exclusion test) by a collagenase

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perfusion technique, as described (Malaveille et al., 1983).

(4) The rec assay was carried out using *B. subtilis* H17 and M45 strains in the absence of S9 (Kada et al., 1980).

(5) Plate incorporation assay, with or without preincubation, in S. typhimurium. The test compound, dissolved in 100 μl (without preincubation) or 50 μl acetone (with preincubation) and 100 μl of an overnight bacterial culture medium ($2-4 \times 10^8$ bacteria), was mixed with medium containing 0 or 100 μl S9, 0 or 2 μmoles $NADP^+$, 0 or 2.5 μmoles glucose 6-phosphate, 4 μmoles $MgCl_2$ and 50 μmoles Sørensen phosphate buffer pH 7.4 (made up to a total volume of 500 μl with 0.15 M KCl in 5 mM Sørensen phosphate buffer, pH 7.4). The mixture was combined with 2 ml of histidine-poor soft agar and plated in duplicate or tetraplicate. In some experiments, the mixture was preincubated for 60 min at 37°C with shaking before the addition of histidine-poor soft agar.

(6) Salmonella / rat hepatocyte assay. Each assay medium consisted of 2 ml of Hanks' BSS-20 mM Hepes buffer, pH 7.4, containing $1-4 \times 10^6$ hepatocytes, about 6×10^8 bacteria, suspended in 100 μl of overnight culture medium and the test compound dissolved in 20 μl acetone. Incubation was carried out at 37°C for 3 h with shaking. An aliquot of 1 ml was plated in duplicate, as described previously (Malaveille et al., 1983).

(7) Fluctuation test. Some of the assays with *S. typhimurium* (Table 1) were carried out using a test procedure adapted from Gatehouse and Delow (1979), Forster et al. (1980) and Hubbard et al. (1984). Media were prepared as follows:

(a) *S9 mixture:* 1 ml of S9 mixture consisted of 100 μl S9, 100 μl 4 mM $NADP^+$, 100 μl 5 mM glucose 6-phosphate, 100 μl 8 mM $MgCl_2$, 400 μl 0.25 M Sørensen phosphate buffer pH 7.4 and 200 μl 0.15 M KCl–5 mM Sørensen buffer.

(b) *Bulk test solution with S9:* 15 ml of the test solution contained 11.6 ml Vogel–Bonner minimal medium, 100 μl 60% w/v glucose, 150 μg biotin, 3 ml of S9 mixture and 0.75 or 1.4 μg/ml histidine for tests with TA100 or TA98 strains, respectively.

(c) *Bulk test solution without S9:* 15 ml of test solution contained 14.6 ml Vogel–Bonner minimal medium, 100 μl 60% w/v glucose, 150 μg biotin and 1.5 or 5 μg/ml histidine for tests with TA100 or TA98, respectively.

For the assay, the bulk test solution — containing about 14×10^5 bacteria (*S. typhimurium* TA100)/ml or about 14×10^6 TA98 bacteria/ml and 10 μl/ml acetone solution of TBTO — was distributed in 50-μl aliquots in 96 wells of a sterile microtitre tray. The trays were incubated overnight at 37°C. Then, 150 μl/well of selective indicator (Vogel–Bonner minimal medium supplemented with 0.4% glucose and 5 μg/ml bromocresol purple) were added. The trays were incubated for a further 4 days at 37°C in plastic bags containing moistened paper towels to maintain a humid environment during incubation. Yellow and turbid wells were scored positive and purple wells negative.

Concurrent positive controls were run with each experiment: 5 μM benzo[*a*]pyrene in the presence of a metabolic activation system increased for TA100 and TA98 the number of positive wells by 5–8- and 2–3-fold, respectively; 0.01 μg/ml of 4-nitroquinoline *N*-oxide increased the number of positive wells for TA98 by 3–5-fold, and 100 μg/ml of methyl methanesulphonate increased the number of positive wells for TA100 by 7–8-fold. The statistical significance of increases in the number of positive wells in treated cells over the corresponding controls was tested by the χ^2 method.

The fluctuation test (in the absence of a metabolic activation system) (Luria and Delbrück, 1943) was used with *K. pneumoniae*, as described earlier in detail (Voogd et al., 1977). TBTO was diluted in dimethyl sulphoxide (DMSO) and added to broth, which was inoculated with the tester strain, a mutant of *K. pneumoniae* ur^- pro^-, requiring uracil and proline for growth. The average spontaneous mutation rate of the strain for streptomycin resistance, including streptomycin dependence, was 0.1676×10^{-9} (S.D. $\pm 0.0304 \times 10^{-9}$ from 26 Expts.).

(II) Forward mutation and mitotic gene conversion in yeast

(1) Preparation of S9 fraction. Livers from Sprague–Dawley male rats or mice (CD-1) were

homogenized in 0.01 M phosphate buffer (pH 7.4, 1.15% KCl). The homogenate (1 g liver per 4 ml buffer) was centrifuged for 10 min at 900 × g and then for 20 min at 9000 × g. Prior to sacrifice, animals received 80 mg/kg/day phenobarbital intraperitoneally for 4 days and a single intraperitoneal injection of 80 mg/kg β-naphthoflavone 2 days before being killed.

The metabolizing system contained per ml: 3 mg $NADP^+$, 6 mg glucose 6-phosphate, 20 μl glucose-6-phosphate dehydrogenase, 500 mM $MgCl_2$ (40 μl), 640 μl phosphate buffer and 300 μl S9 fraction.

(2) Forward mutation in Schizosaccharomyces pombe. Suspensions of *S. pombe* P1 cells were incubated with TBTO for 16 h at 32°C, in the presence or absence of a mouse liver S9 activation system. The cells were then diluted, plated, incubated and finally scored for the presence of white colonies of *ade* mutants, as described by Barale et al. (1979). TBTO solutions were prepared in ethanol; the final concentration of ethanol in the incubate did not exceed 5% by volume. Methyl methanesulphonate and cyclophosphamide were used as positive controls.

(3) Mitotic gene conversion in Saccharomyces cerevisiae D4. Suspensions of *S. cerevisiae* D4 cells were incubated with TBTO for 16 h at 32°C in the presence or absence of a mouse liver S9 activation system. The cells were diluted, plated, incubated and finally scored for viable and convertant colonies at the *ade* and *trp* loci, as described by Zimmermann et al. (1975). TBTO solutions were prepared in ethanol; the final concentration of ethanol in the incubation mixtures did not exceed 5% by volume. Hycanthone and cyclophosphamide were used as positive controls.

(III) Sex-linked recessive lethal mutations in Drosophila melanogaster

Both feeding and injection experiments were carried out with adult Drosophila using the X-linked recessive lethal assay (Würgler et al., 1984). The test substance was dissolved in a mixture of 8% Tween 80, 8% ethanol and 5% sucrose and fed to tester males (strain Berlin K) for 48 h, according to a technique described earlier (Zijlstra and Vogel, 1984). 2-day-old tester males were starved in empty vials for 3–4 h, to ensure immediate uptake of test solution at the start of treatment. For injection experiments, the test substance was prepared as an emulsion containing 7.5% cottonseed oil, 0.6% lecithin, 0.15% pluronic F-68 and 0.7% NaCl. After oral treatment males were always mated after termination of treatment, whereas after injection treatment they were allowed to recover for 24 h. All cultures and treatments were maintained at 25°C.

For induction of X-linked recessive lethals, the standard procedure was to mate 1 Berlin K male to 4 virgin females of genetic constitution $In(1)sc^{SIL}$ $sc^{8R} + S$, sc^{S1} sc^8 w^a *B (Basc).* The progeny were fractionated into 3 consecutive broods at 2- or 3-day intervals for a total of 7 days. F_2 cultures with more than one wild-type (Berlin K) male were considered to be nonlethals. Further testing in F_3 was carried out to confirm that a suspected culture carried a recessive lethal mutation.

(IV) Gene mutations in Chinese hamster V79 cells and L5178Y mouse lymphoma cells

(1) In microsome-mediated assays (Table 4, Expts. 1 and 2), Chinese hamster V79 cells (overnight culture after plating 10^6 cells/60-mm dish) were incubated for 3 h in 2.5 ml of a mixture containing 2% w/v of a 15000 × g liver supernatant (S15) from phenobarbital- and 5,6-benzoflavone-pretreated Sprague–Dawley rats (Matsushima et al., 1976) in the presence or absence of cofactors ($NADP^+$, $NADPH^+$, glucose 6-phosphate, Mg^{2+}) and TBTO dissolved in DMSO (final concentration, 0.8% v/v); this was followed by postincubation in fresh culture medium for 2–3 h and plating to determine induced cytotoxicity and 8-azaguanine- (20 μg/ml) and ouabain- (1 μmole/ml) resistant mutants. The drugs were added 48–72 h after plating (Kuroki et al., 1977).

(2) In cell-mediated assays (Table 4, Expts. 3–10) (Huberman and Sachs, 1974), irradiated hamster embryo cells at secondary culture and nonirradiated epidermal cells of mice (C3H, newborn) and humans (isolated from a 24-year-old male) were used (Hosomi et al., 1982; Kuroki et al., 1980, 1982). V79 Chinese hamster cells (3×10^5/dish) were plated on confluent sheets of these

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cells, and, 5–6 h later, TBTO dissolved in DMSO (final concentration, 0.5% v/v) was added for 48 h, followed by plating to determine induced cytotoxicity and 8-azaguanine- and ouabain-resistant mutant colonies.

(3) In assays using Chinese hamster V79 cells (Table 4, Expts. 11 and 12), these were cultured in Ham's F-10 medium without hypoxanthine, supplemented with 15% newborn calf serum. After plating 5×10^6 cells/150-cm^2 flask overnight cultures were incubated for 2 h at 37°C in either a mixture containing rat S9 (as described in Section I.2) and cofactors in phosphate-buffered saline or medium without serum, to which TBTO diluted in ethanol (final concentration, 1% v/v) was added at various concentrations. After treatment, a few cells per group were seeded for determination of induced cytotoxicity, and $2–3 \times 10^6$ cells were cultured for an expression time of 7 days with subculture at day 3. At day 7, cells from each group were seeded for determination of HPRT-deficient mutants in 6-thioguanine (5 μg/ml)-containing medium (10^5 cells/90-mm dish, 10 dishes) and for cloning efficiency.

(4) In assays with L5178Y mouse lymphoma cells (Table 4, Expts. 13 and 14), heterozygous for thymidine kinase ($TK^{+/-}$), without S9, cells were cultured in Ham's F-10 medium without hypoxanthine but supplemented with 15% newborn calf serum. Cells at a density of 2×10^6/ml were treated for 2 h at 37°C in medium without serum, to which TBTO diluted in ethanol (final concentration, 1% v/v) was added at various concentrations. After treatment, a few cells per group were seeded for determination of induced cytotoxicity, and 1.5×10^7 cells per group were propagated and subcultured daily during the expression time: 3 days for $TK^{-/-}$ mutants and 7 days for $HPRT^-$ mutants. At the end of the expression times, cells from each group were seeded for cloning efficiency, and 3×10^6 cells from each group were seeded for determination of $TK^{-/-}$ mutants in BUdR (50 μg/ml)-containing medium (10^4 cells/ml) or of $HPRT^-$ mutants in 6-thioguanine (5 μg/ml)-containing medium (2×10^4 cells/ml).

(V) Induction of sister-chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells

CHO cells, grown in Ham's F-10 medium, were incubated with TBTO in the presence of BUdR (3 μg/ml) for 24 h; colchicine was added. In the presence of rat S9 mix, treatment was for only 3 h, after which the cells were incubated a further 24 h in fresh medium supplemented with 3 μg/ml BUdR. In both experiments colchicine was present for the final 3 h. Cultures treated with TBTO either in the presence or absence of an S9 system were trypsinized, treated with hypotonic medium and fixed, and then prepared as air-dried slides. The latter were stained using the FPG method, and 25 metaphases per culture were scored for SCE. TBTO solutions were prepared in ethanol; the final concentration of ethanol in treatment medium did not exceed 1% by volume. Cyclophosphamide was used as a positive control.

(VI) Chromosomal aberrations in CHO cells

Chinese hamster ovary cells, grown in Ham's F-10 medium, were treated with TBTO in the presence or absence of rat S9 for 3 h. The cultures were then washed and allowed to recover prior to harvesting 8, 15 and 24 h after the commencement of treatment. Colchicine was present for 3 h before harvesting. Air-dried slides were prepared, stained with 3% Giemsa in buffer and fixed.

When possible, 100 well-spread metaphases per culture were scored for aberrations. 5 dose levels of TBTO were assayed, of which at least 3 were scored for aberrations.

TBTO solutions were prepared in DMSO. Cyclophosphamide was used as a positive control (data not shown).

(VII) Inhibitory effects on intercellular metabolic cooperation

In brief the method described by Yotti et al. (1979) and Umeda et al. (1980) was used. One hundred 6-thioguanine (6TG)-resistant mutant cells and 2.5×10^5 wild-type Chinese hamster V79 cells were inoculated into 6-cm plastic dishes in 4-ml amounts. After a 4-h incubation, 1 ml of medium was added to each dish with 25 μg/ml of 6-thioguanine and various concentrations of TBTO. Cultures of 6-thioguanine-resistant cells alone were also prepared for testing the toxic effect of TBTO. Each group consisted of 4 dishes, and cells were cultured for 7 days for colony formation. Data were expressed as percent plating

efficiency of experimental cultures relative to that of the control 6-thioguanine-resistant cells alone.

(VIII) Induction of micronuclei in the bone marrow of mice

For the micronucleus test, mice (strain BALB/c, aged 6–8 weeks, obtained from Kuopio University Animal Centre, Finland) were kept under standard housing conditions for 2 weeks before the beginning of the experiment. On the basis of data in the literature and a small pilot study, 2 doses of TBTO — 60 mg/kg bw and 30 mg/kg bw — were selected and administered by a single oral treatment (by gavage in olive oil).

Each test group consisted of 5 males and 5 females. Two intervals — 30 h and 48 h — between treatment and killing of the animals were used. Cyclophosphamide (15 mg/kg bw; intraperitoneally) was used as the positive control, and olive oil (0.02 ml/g bw; by gavage) as the solvent control.

The animals were killed 30 h or 48 h after the treatment by cervical dislocation. Both femurs were removed, and the bone marrow was aspirated with fetal calf serum and prepared as described by Schmid (1975). Preparations were stained as described by Penttilä et al. (1980).

The slides (2 per animal) were coded and analysed by one person: 1000 polychromatic erythrocytes (PCEs) and 1000 normochromatic erythrocytes (NCEs) were scored for each animal, and the ratio of the two was determined simultaneously.

(IX) Toxic effects of TBTO in adult mice and on prenatal development

(1) NMRI mice (Zentralinstitut für Versuchstiere, Hanover, FRG) were kept under spf conditions and fed Altromin 1324 and tap water ad libitum. TBTO was dissolved in olive oil and administered by stomach tube. Animals were mated for 2 h. The 24-h period following successful mating ('plug day') was considered to be day 0. Pregnant animals were sacrificed on day 18.

(2) Light and electron microscopy. After treatment with 30 or 110 mg TBTO/kg bw on day 10 of gestation, the embryos, livers and kidneys of animals were removed 24 and 48 h later, fixed in Karnovsky's solution (3% paraformaldehyde plus 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2), dehydrated in the acetone series and embedded in Mikropal. Sections were prepared using Reichert and LKB microtomes and contrasted with uranyl acetate/lead citrate. For light microscopic inspection, 1-μm sections were stained with alkaline Giemsa solution (1%) at 60 °C for 1 min.

(3) Determination of tin content. Pregnant mice (day 11) were sacrificed 2–48 h after oral treatment with TBTO (110 mg/kg bw). The organs were removed and frozen in liquid nitrogen, and approximately 20 mg of the freeze-dried tissues and sera were ashed overnight in a low-temperature asher at 1 Torr O_2. The residue was dissolved in 200 μl 0.1 N HCl. Tin was measured in an atomic absorption spectrophotometer (Perkin-Elmer, Model 300) with a graphite furnace (HGA 72). After pipetting 20 μl of test probe and 20 μl 0.2 M NH_4OH into the graphite furnace, the probe was dried at 100 °C for 20 sec, ashed at 750 °C for 20 sec, atomized at 2700 °C for 10 sec and measured at 224.6 nm. Calibration was performed in the same way with $SnCl_2 \cdot H_2O$ (pro analysi), dissolved in 0.1 N HCl. All solutions were made with ultrapure water.

Results

Results are presented and summarized in Tables 1–10 and additional raw data are shown in Tables A1–A11 of the Appendix.

(I) DNA damage and mutations in bacteria

In the absence of metabolic activation, TBTO had no mutagenic activity in *K. pneumoniae* in the fluctuation test, when tested up to limit of its solubility (Table 1). These high concentrations caused some inhibition of growth. Similarly, in the absence of a metabolic activation system the inhibition zones with 58.5 mg TBTO/plate in the rec assay with *B. subtilis* strains H17 rec$^+$ and M45 rec$^-$ were both 14 mm indicating no DNA-damaging activity (Table 1).

In the plate incorporation test, TBTO showed no mutagenic activity in *S. typhimurium* strains TA100, TA98, TA97, TA1535, TA1538 and

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TABLE 1

TESTS FOR DNA DAMAGE AND MUTATION IN BACTERIA AND YEASTS

Expt No.	Genetic indicator	Endpoint (test)	Metabolic activation system	Length of treatment (h)	Concentration range	Results		Toxicity	Remarks; number of tables in Appendix
						TBTO-treated	Untreated		
1	*B. subtilis* H17, M45	Differential DNA damage/repair (*rec* assay)	none	24	58 mg/plate	Zones of growth inhibition (mm)		–	Highest concentration tested
						+ 14	14		
						Mutation rate $\times 10^9$ (at 1170 μg/ml)		Growth inhibition at high concentrations	
2	*K. pneumoniae*	Revertant (hr^- pro^-) mutations (fluctuation test)	none	20	11.7–1170 μg/ml (19.7–1970 μM)	0.077–0.082	0.119–0.157		Table A1
3	*S. typhimurium*	Reverse (his^-) mutations (plate assay)				Revertants/plate (at 1 μg/plate)		Toxicity at high concentrations	
	TA98		rat liver S9	48	10^{-4}–10^3 μg/plate	20	26		Table A2
			none			26	15		
	TA100		rat liver S9		10^{-5}–10^3 μg/plate	151	131		Table A2
			none			86	126		
						Revertants/plate (at 10 μg/plate)			
4	TA1530		rat liver S9		2.5–500 μg/plate	14	14		Table A3
			none			16	20		
	TA1535		rat liver S9			16	20		
			none			6	12		
	TA97		rat liver S9			154	157		
			none			5	51		
	TA98		rat liver S9			62	52		
			none			24	31		
	TA100		rat liver S9			120	130		
			none			13	104		

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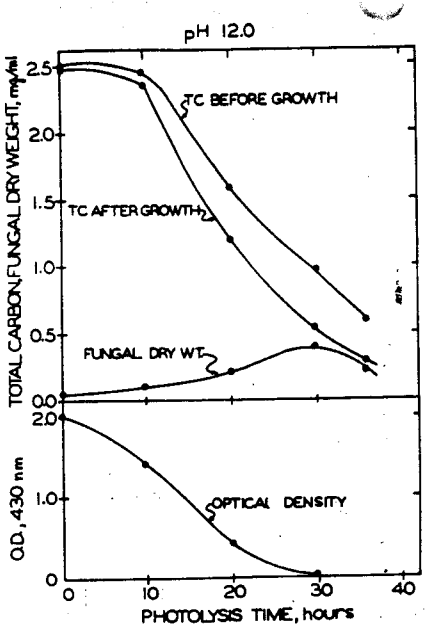

Fig. 3—Aspergillus sp. growth response to initial pH 12.0, 0.5% w/v, photooxidized sodium ligninsulfonate.

also be considered for use with other recalcitrant materials.

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Arsenic Toxicity to Cotton and Soybeans[1]

Lloyd E. Deuel and Allen R. Swoboda[2]

ABSTRACT

Arsenic toxicity levels for 'Lankert' cotton (Gossypium hirsutum L.) and 'Patterson' soybeans (Glycine max L. merr.) were determined in a greenhouse experiment using an Amarillo fine sandy loam and a Houston Black clay. The treatments ranged from 28 to 280 kg As/ha in the Amarillo soil and from 56 to 560 kg As/ha in the Houston Black soil. The vegetative cotton yields were significantly decreased below the checks at the 56 and 280 kg/ha rates of applied As in the Amarillo and Houston Black soils, respectively. Soybeans were found to be more sensitive to As than cotton. Vegetative soybean yields were significantly decreased at the applied rates of 28 kg As/ha in the Amarillo soil and 168 kg As/ha in the Houston Black soil. Yield limiting As concentrations in plant tissue were 4.4 ppm and greater in cotton, and 1 ppm and greater in soybeans. Water soluble As levels of 8 ppm in the Amarillo soil and 28 ppm in the Houston Black soil were required for a significant decrease in the vegetative growth of cotton. The water soluble As levels detrimental to soybeans were 3 and 12 ppm in the Amarillo and Houston Black soils, respectively.

Additional Index Words: soluble arsenic, arsenic extraction, arsenic toxicity, cotton, and soybeans.

Arsenicals have been used in agriculture to combat "pests" for more than a century. More recently organic arsenicals have been introduced for use as herbicides. Their low cost and effectiveness have been instrumental in keeping arsenic on the market. However, they are pollutants by today's standards and their continued use can only be justified from a benefit-risk viewpoint.

In 1922, Stewart (12) reported that 75 ppm As in a calcareous Bench loam caused only slight detrimental affects to the more As sensitive plants, but that 25 ppm As in the soil appeared to have stimulated them. From these results he concluded that small quantities may be beneficial to some plants.

Albert and Arndt (1) found that 1 ppm of soluble As retarded the top and root growth of cowpeas. Machlis (8) used Sudan grass and bush beans as the indicator crops

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Table 1—Correlation coefficient[illegible] yield vs. extractable arsenic

Treatment	Average yield	
	Cotton	Soybeans†
kg As/ha	g/pot	
	Amarillo soil	
0	2.91 a*	(8.75) a*
28	2.27 ab	(4.80) b
56	1.39 bc	2.95 c
84	1.20 cd	1.31 cd
112	0.94 cde	0.31 d
140	0.94 cde	
168	0.86 cde	
196	0.32 de	
224	0.34 de	
252	0.17 e	
280	0.15 e	
	Houston soil	
0	2.65 a	(10.45) a
56	2.69 a	(7.55) ab
112	2.54 ab	(7.82) ab
168	2.33 ab	(6.25) bc
224	1.89 abc	(4.23) bcd
280	1.60 bc	3.39 cd
336	1.36 c	1.67 d
392	1.40 c	1.87 d
448	1.62 bc	1.29 de
504	1.28 c	0.05 e
560	1.25 c	0.25 e

* Any two numbers in the same column not followed by the same letter are significantly different at the 0.05 level.
† A number followed by ( ) indicates the plants set fruit.

to demonstrate that As can accumulate in plant tops. However, several studies indicated that the possible poisoning of man and animals by ingesting contaminated plants was remote, since the levels of As toxic to man and animals were also toxic to plants (1, 5, 9, 13). Recently Jacobs et al. (4) reported that, with the exception of potato peelings, As levels in the edible portions of vegetables grown on a soil treated with 180 kg As/ha, were below the tolerance limit of 2.6 ppm. However, the yields were depressed considerably at this rate.

The objective of the present study was to determine the levels of As that adversely affect the vegetative growth of cotton and soybeans grown on two Texas soils in the greenhouse.

MATERIALS AND METHODS

An Amarillo fine sandy loam and a Houston Black clay were chosen for this study because they are predominant soil types of the major cotton stripping areas of Texas. Arsenic acid is the primary desiccant used to aid in the stripping of cotton in these areas.

A randomized block design with 11 treatment rates and 2 replications was used. A 2-kg sample of each soil was placed in 44 plastic pots. Eleven As treatments in quadruplicate were made on each soil as shown in Table 1. Stock mixtures of As were prepared by mixing As_2O_3 with a sample of each soil to give final mixtures of 2 mg As/g soil and 10 mg As/g of soil. A prescribed amount of a stock mixture was added to each pot to give the desired treatment. A portion of the soil previously weighed was removed before the addition of the stock mixtures so that the final weight in each pot was 2 kg. Stock mixtures provided a method whereby the As could be applied in sufficient bulk to aid in the mechanical mixing of the As into the soil. Mixing was accomplished with a "V" shaped tumbling mixer.

The soils were placed in a greenhouse and brought to field capacity with distilled H_2O. Field capacity was 15% and 28%, by weight, for the Amarillo and Houston Black soils, respectively. The soils were allowed to dry for 4 or 5 days and then brought back to field capacity. This cycle was continued for 2 months to allow for complete reaction of the As with the soil. After 2 months the pots were subdivided at random into 2 groups such that each level of As was represented in duplicate for both soils.

One group of pots was seeded with 'Lankert' cotton (*Gossypium hirsutum* L.) and the other with 'Patterson' soybeans (*Glycine max* L. merr.). The planted pots were brought to field capacity with distilled water and checked daily throughout the 6-week growing period to maintain the soils at field capacity. Each crop received an application of 112 kg N/ha applied as NH_4NO_3 in solution when the plants were 2 weeks old. No other fertilizer was applied. The cotton and soybeans were thinned to 2 and 5 plants/pot, respectively, just prior to the application of N.

The crops were harvested at 6 weeks by excising the plant at the soil line. They were then placed in a forced draft oven and dried at 105C for 24 hours. Oven dry weights of the plants in each pot were determined to give the yield per pot. Since some of the soybeans had set fruit a note was made of this while recording yield data.

Arsenic contents of the plants grown in the greenhouse were determined by the method described by Liederman, Bowen, and Milner (7), with a slight modification of the acid used to digest the plants. A ternary acid mixture consisting of 200 ml conc. H_2SO_4, 1000 ml conc. HNO_3, and 400 ml 70% $HClO_4$ was employed in the digestion procedure.

After the plants were harvested, soil samples were taken from each pot and extracted with hot (95C) distilled H_2O, 1*N* NH_4Cl and 0.5*N* HCl. Three, 15-ml increments were used to facilitate the extraction of about 5 g of soil. Alternate shaking and centrifugation was used to separate the supernatant liquid from the soil. The extract was brought to volume in a 50-ml volumetric flask with distilled H_2O. A suitable aliquot was taken for the determination of As using the arsine evolution procedure (7).

Analyses of variance were determined on the yield data for both cotton and soybeans on the two soils at the corresponding As treatment levels. A Tukey D test was employed to determine which yields were significantly different. Correlation coefficients were also determined between mean yields of each crop and the extractable soil As levels.

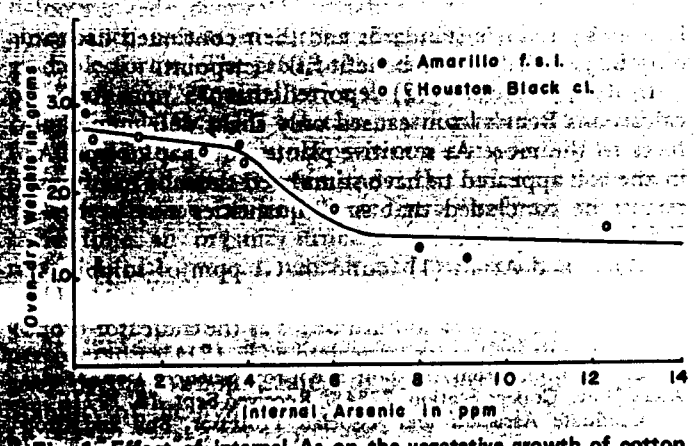

Fig. 1—Effect of internal As on the vegetative growth of cotton.

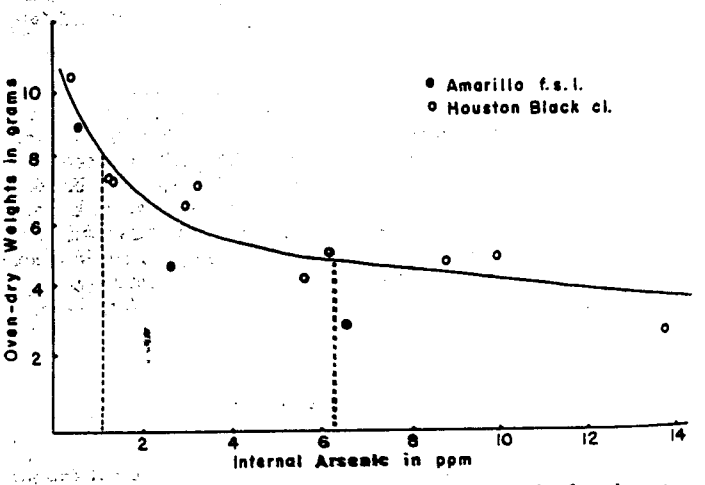

Fig. 2—Effect of internal As on the vegetative growth of soybeans.

RESULTS AND DISCUSSION

The yields for the cotton grown on the Amarillo and Houston Black soils, were reduced by As applications (Table 1). The analyses of variance indicated that the yields decreased with increased As at the 1% level of significance. The cotton grown on the Amarillo soil was more seriously affected at the lower rates of applied As than the cotton grown on the Houston Black soil. Vegetative yields were significantly reduced below the check at the 56 kg/ha treatment in the Amarillo soil. A significant yield reduction was observed for cotton plants grown on the Houston Black clay at the treatment level of 280 kg/ha.

The vegetative yields for the soybeans grown on the Amarillo and Houston Black soils are given in Table 1, along with the As levels which inhibited fruiting. Soybeans generally require more than 6 weeks to set fruit even in the greenhouse. However, the short daylength encountered induced early flowering and fruitage of the Patterson variety of soybeans. The pods formed were about 5 cm long. Analyses of variance suggested the vegetative soybean yields decreased with increased As at the 1% level of significance. Soybeans were found to be more sensitive to As than cotton. Yield decreases were observed at As concentrations of 28 and 168 kg/ha in the Amarillo and Houston Black soils, respectively. The soybeans did not set fruit beyond the 28 kg As/ha treatment in the Amarillo soil. However, in the Houston Black clay fruiting was observed at all treatments below 224 kg As/ha in one replication and 280 kg As/ha in the other.

There was a slight increase in the vegetative yields of both crops grown in the Houston Black clay; although, the increase was not significant. This occurred at the 56 and 112 kg/ha rates of applied As for the soybeans and cotton, respectively. The increase is possibly due to the release of some phosphorus by the added arsenic. Several workers have demonstrated that a replacement for one by the other from soil complexing agents can occur (2, 3, 6, 10, 11).

The data for the averaged vegetative cotton yields for both soils and the plant As contents are plotted in Fig. 1. The yields and internal As contents for the soybeans grown on both soils are shown in Fig. 2. The tissue yields

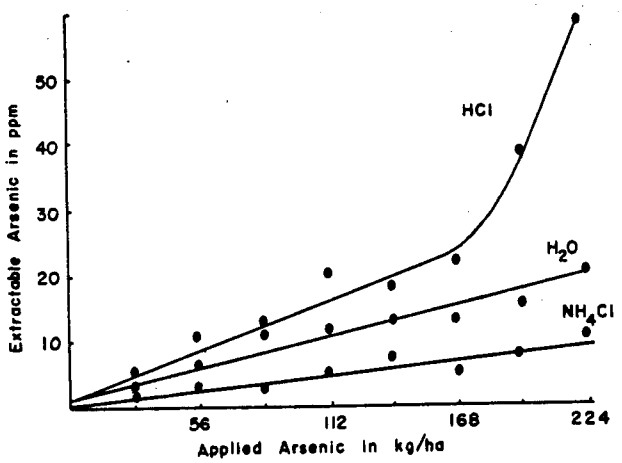

Fig. 3—Extractable As corresponding to the applied rates in an Amarillo fine sandy loam.

Table 2—Effect of arsenic on the fruiting and vegetative growth of cotton and soybeans

Soil	Crop	Extractant* H_2O	HCl	NH_4Cl
Amarillo	Soybeans	.943	.915	.914
Houston		.968	.931	.988
Amarillo	Cotton	.951	.830	.960
Houston		.954	.895	.918

* All of the correlation coefficients are significant at the 0.05 level.

from some of the treatments were not sufficient for chemical analysis and several of the samples were lost in the initial digestion procedure. The curves indicated that As exerted the same toxic effect once taken up by the plant, regardless of the soil. An internal As concentration exceeding 4.4 ppm caused a significant decrease in the vegetative growth of cotton in both soils. It is interesting to note that the decrease was about 50% of the vegetative growth, and that cotton demonstrates a threshold limit for As. An internal As concentration of 1 ppm induced a significant decrease in the vegetative growth of soybeans, but did not prevent fruiting. No fruit was set in the soybeans when the internal As concentration exceeded 6.2 ppm. However, fruit was set and maturing at internal levels exceeding the tolerance limit of 2.6 ppm As established for many vegetable crops (4).

Since As toxicity to plants is directly related to the soluble level in the soil, a measure of the soluble As content should be more informative than the total As. The data for the soluble As, determined with 3 extractants at the different levels of applied As, is graphically depicted in Fig. 3 for the Amarillo soil and Fig. 4 for the Houston Black clay soil. Correlation coefficients determined for the averaged yields of both crops and the As extracted by each solution are given in Table 2. Although the correlation coefficients were all significant at the 5% level or better, the curves shown in Fig. 3 and 4 suggest that hot (95C) distilled H_2O is the preferable extractant for soluble As in the Amarillo and Houston Black clay soils. The hot H_2O extracted similar quantities of As for both soils at comparable rates of applied As. The other extractants varied between soils. Water soluble As levels of 8 ppm in the Amarillo and 28 ppm in the Houston Black clay were required for a significant decrease in the vegetative growth of cotton. The corresponding values for soybeans were 3 and 12 ppm As in the Amarillo and Houston Black soils, respectively. The toxic levels may have been slightly exaggerated due to the high root density, uniform As distribution in the soil, and the higher soil temperatures ob-

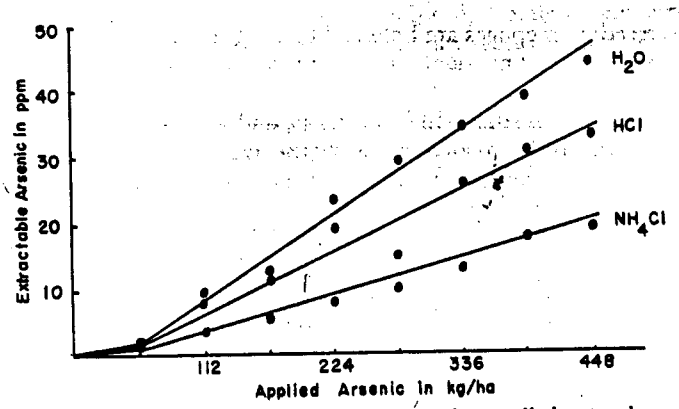

Fig. 4—Extractable As corresponding to the applied rates in a Houston Black clay.

tained in the greenhouse pots. The higher soil temperature would tend to induce a greater soluble level of As in the soil.

The data indicates that a narrower range between tolerable levels of soluble As and the levels which effect yields exists in the Amarillo soil than in the Houston Black soil. However, a greater hazard of crops exceeding the allowable As level probably exists in the Houston Black clay due to the more subtle effect of soluble As in this soil.

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Water Soluble Organic Substances Leachable from Feedlot Manure[1]

A. R. Mosier[2], K. Haider[3], and F. E. Clark[2]

ABSTRACT

Water extracts of feedlot manure and soil and of grassland soil were compared with water from shallow wells near to and remote from feedlots and with water from a local river. Thin-layer and column chromatography failed to reveal water-soluble organics unique to feedlot ground waters. Free phenolics could readily be leached from feedlot manure and from the upper few centimeters of feedlot soil, but only trace amounts of low-molecular-weight organics were encountered in the ground water samples examined. The major organic constituents in all the well waters appeared to be polymerized materials. Mildly reductive degradation of the organic polymers in leachates prepared from feedlot manure, feedlot soil, and grassland soil yielded identifiable phenolics; the same procedure failed to reveal phenolics in ground waters. It was concluded that there is no uniform or continuing movement of organic material from feedlots through the soil profile to the ground water under the feedlot sites involved.

Additional Index Words: phenolics, ground water.

Feedlot seepages are believed to contribute to the organic and mineral nutrient enrichment of lakes and streams and to produce high rates of oxygen demand, bad tastes and odors, undesirable algal growths, and wide-ranging effects on the biota associated with the water bodies (12, 15). The urea excreted in animal urine is rapidly transformed to ammonia and this, in turn, may undergo volatilization or be microbially oxidized to nitrite and nitrate. Ground water in the vicinity of feedlots frequently is high in nitrate (1, 17). However, little is known about the fate in soil of the water-soluble organic products of animal excretion other than urea and the extent to which such products can be detected in soil leachates. The current work was undertaken to compare the water-soluble organic material of feedlot manure and soil with the organic materials found in leachates of grassland soil and in ground water in the vicinity of and remote from feedlots.

Surface and ground waters have been found to contain traces of a wide variety of organic compounds. Lamar and Goerlitz (10) concluded that much of the organic matter in surface waters consists of a mixture of carboxylic acids having aromatic and olefinic unsaturations. Middleton et al. (12) isolated a large number of organic compounds, mostly in parts per billion concentrations, from surface waters variously contaminated with petrochemical and industrial wastes. Schnitzer and Des Jardins (16) analyzed the aqueous leachate collected in a field lysimeter from a humic podzol and found that about 87% of the dry, ash-free weight of the leachate was fulvic acid, with the remainder consisting mostly of polysaccharides and nitrogenous compounds.

Usually only low yields of phenolic substances are extractable from soils and peats (6). Christman and Ghasemi (5) observed trace amounts of phenols in water extracts of soils and sawdusts of different tree species. Most of the water-extractable material appeared to be polymeric, from which different phenols were released upon oxidative degradation with copper oxide. Schnitzer and Des Jardins (16) obtained no free phenols in the

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