Pharmacokinetic evidence for an enterohepatic circulation in a patient treated with cis-dichlorodiammineplatinum (II)

J.B. Vermorken\textsuperscript{X}, W.J.F. van der Vijgh\textsuperscript{XX} and H.M. Pinedo\textsuperscript{X, O}

University Hospital of the Free University
\textsuperscript{X} Department of Oncology
\textsuperscript{XX} Research Laboratory
De Boelelaan 1117, Amsterdam, The Netherlands
\textsuperscript{O} Netherlands Cancer Institute

\textbf{ABSTRACT}

Platinum concentrations in plasma were measured by atomic absorption spectrometry during and after a 15 min intravenous infusion of cis-dichlorodiammineplatinum (II) (cis-platinum) at a dose of 100 mg/m\textsuperscript{2}. Plasma concentrations of platinum declined biexponentially (t\textsubscript{1/2}\textsuperscript{X}=21.3 min, t\textsubscript{1/2}\textsuperscript{P}=6.2 days). A small peak was noticed at 3.5 hrs after the end of the infusion, indicating a renewed influx of platinum in the plasma compartment. This phenomenon is strongly suggestive for the presence of an enterohepatic circulation.

\textbf{INTRODUCTION}

Cis-platinum is an antitumor agent which has been available for clinical trials since 1972. The drug appears to be active against a variety of tumors including ovarian cancer (Williams and Whitehouse, 1979). Even in patients with
ovarian cancer resistant to conventional chemotherapy cis-platinum has proven to be effective, in particular when used in high dosages (Wiltshaw, 1978).

The major excretory pathway of cis-platinum is through the kidneys (Lange et al, 1973, Smith and Taylor, 1974). Biliary excretion of platinum has been demonstrated in animals (Litterst et al, 1976, LeRoy et al, 1979, De Simone et al, 1979). In dogs only small quantities of platinum have been detected in the bile during observation periods up to 12 days (Litterst et al, 1979, LeRoy et al, 1979) which indicates that biliary excretion is not an important route of elimination in this species. In rats up to 2% of an i.v. dose of $^{195m}$Pt-cis-platinum was excreted in the bile during the first 24 hours (De Simone et al, 1979). Although the existence of an enterohepatic circulation of cis-platinum has been suggested in man (Smith and Taylor, 1974), this has never been proven. We observed repeatedly a small peak in the plasma platinum concentration curve during the initial hrs following a cis-platinum infusion in our patients. By using a very intensive sampling scheme, applied in the present case, we have been able to obtain additional supportive data for the existence of this phenomenon.

**MATERIALS AND METHODS**

**Patient data**

In 1978 a papillary adenocarcinoma of the left ovary stage
Figure 1 - Platinum levels in plasma after a 15 min i.v. infusion of 175 mg cis-platinum. The dotted lines represent the best linear fit for the initial rapid disposition phase. The open circles represent the measured plasma levels minus the predicted values from back-extrapolation of the elimination line.

Squares method within the time intervals of 0-30 min and 1-10 days respectively. The levels from 0.5-21 hrs following the cis-platinum infusion do not fit to these lines. In order to obtain the exact deviation, the extrapolated values of the $\alpha$- and $\beta$-phase during 0.5-21 hrs after the cis-platinum infusion were subtracted from the levels actually measured. The deviations between measured and calculated values are consistently positive, as shown in figure 2. From 0.5 hr after the end of infusion a rapid increase is observed reaching a peak value of 0.5 $\mu$g Pt/ml
IV (FIGO-classification) was diagnosed in a 44 year old female. She was treated with L-phenylalanine mustard for one year. Because of progressive disease the patient was admitted to the Department of Oncology for treatment with cis-platinum. At that time the results of renal function tests and liver function tests were within normal ranges. There were no signs of ascites or pleural effusion.

Administration of drugs
During 4 hrs prior to cis-platinum administration the patient received one liter of normal saline. For cis-platinum therapy vials of Platinol® were used. Each vial contained 10 mg of cis-platinum, 90 mg of sodium chloride and 100 mg of mannitol. After reconstitution with sterile water a drug concentration of 1 mg cis-platinum per milliliter was achieved. The drug was given intravenously at a dose of 100 mg/m² (total dose 175 mg) in 15 min. Following cis-platinum administration the patient received 4 liters of normal saline over a 24 hr period. During and after cis-platinum therapy the only other drug used was metoclopramide.

Sampling and assay
Blood samples were taken immediately prior to administration of cis-platinum, at the end of the infusion, and 5, 10, 15, 30, 60, 90, 120, 150, 180, 210, 240 min. and 6, 8, 21, 24 hr following the administration of cis-platinum. After the first 24 hr observation period blood samples
were taken daily for 10 days with 24 hr intervals. All samples were centrifuged immediately. The plasma was removed and stored at -30°C. The plasma samples were thawed just before analysis and diluted (1:1) with a solution containing 0.4 N HCl and 0.15 N NaCl. The samples were then analyzed for total platinum concentration by atomic absorption spectrometry using a Perkin Elmer atomic absorption spectrophotometer No. 373. The conditions were as follows: 40 sec. drying at 125°C, 120 sec. thermal decomposition at 1400°C and 10 sec. atomization at 2650°C.

RESULTS

A semilogarithmic plot of the plasma platinum concentrations versus time is shown in figure 1. At the end of the infusion a peak concentration of 8.69 µg Pt/ml plasma was reached. The first decline of the curve, representing the initial rapid distribution and elimination, was followed by a small peak at 3.5 hrs after the end of the infusion. From about 20 hrs after this small peak onwards a gradual decrease of plasma platinum concentration was observed due to the elimination of the drug. The biexponential decline in plasma platinum levels is in accordance with a two compartment open model.

The corresponding pharmacokinetic parameters were calculated (Gibaldi and Perrier, 1975) and tabulated in table 1. The monoexponentially declining lines of distribution and elimination could be determined accurately with the least
TABLE 1 - Pharmacokinetic parameters of cis-platinum assuming a two compartment open model

\[ \begin{align*}
    t_{1/2} &= 21.3 \text{ min (} r = -0.998) \\
    A &= 6.65 \text{ µg Pt. ml}^{-1} \\
    k_{10} &= 0.33 \times 10^{-3} \text{ min}^{-1} \\
    t_{1/2} &= 6.2 \text{ days (} r = -0.994) \\
    B &= 2.01 \text{ µg Pt. ml}^{-1} \\
    k_{12} &= 24.6 \times 10^{-3} \text{ min}^{-1} \\
    k_{21} &= 7.6 \times 10^{-3} \text{ min}^{-1} \\
    V_C &= 13.2 \text{ liter} \\
    V_T &= 42.6 \text{ liter}
\end{align*} \]

FIGURE 2 - Semilogarithmic plot of the deviation of the measured and expected plasma platinum levels versus time. \( C_T^\beta \) = measured plasma platinum level at time \( t \), \( C_T^\alpha \) = calculated plasma platinum level at time \( t \) by back extrapolation of the elimination line, \( C_T^\gamma \) = calculated plasma platinum level at time \( t \) by extrapolating the initial rapid disposition line.
at 3.5 hrs. Following this peak a slow decline is observed until 24 hrs after the infusion. These findings are very suggestive for the existence of an enterohepatic circulation of platinum species in this patient.

**DISCUSSION**

In order to obtain more information about the background of the peak observed in the plasma decay curve of platinum in previous patients, we applied in the present case a very intensive sampling scheme after a short term infusion of cis-platinum.

The plasma platinum concentrations measured, declined in a biphasic manner with a half-life for the initial rapid disposition phase \( t_{1/2\alpha} \) of 21.3 min and a half-life of elimination \( t_{1/2\beta} \) of 6.2 days. The \( t_{1/2\alpha} \) is in good agreement with those found in some previous studies (DeConti et al, 1973, Gormly et al, 1979). However, the \( t_{1/2\beta} \) is somewhat longer than that found in the same studies (58-73 hrs). This difference may be explained by the positive deviation of plasma platinum levels during the first 24 hrs and indicates that the \( t_{1/2\beta} \) should be calculated after the initial 24 hrs following the administration of the drug.

The calculated volumes of distribution for the central compartment \( V_C = 13.2 \) liter) and total compartment \( V_T = 42.6 \) liter) suggest that the drug is distributed mainly in the
extracellular and intracellular fluids. At the same time it could mean the absence of "deep" tissue in the peripheral compartments. However, these facts are not in agreement with the observation that only 50% of the administered dose is excreted in the urine during four weeks (unpublished data), while only a small amount is thought to be excreted in the faeces (Smith and Taylor, 1974). Further studies are needed to solve this discrepancy.

The difference between the measured plasma platinum concentrations and the calculated levels from $t_{1/2\alpha}$ and $t_{1/2\beta}$ strongly suggest the presence of an enterohepatic circulation. Animal studies confirm this assumption. There is a strong indication that the platinum found in the faeces originates from the bile (DeSimone et al, 1979). In the latter study $^{195m}$Pt was detected in bile during the first minute of bile flow, following the administration of $^{195m}$Pt-cis-platinum. Bile excretion curves were similar to the urinary excretion curves, showing a rapid initial excretion phase, followed by a slow protracted phase. Considering the given data more than 50% of the $^{195m}$Pt appearing in the bile during the initial 24 hrs was actually excreted during the first 4 hrs. If this platinum is rapidly absorbed in the small bowel a second influx of the drug will occur in the plasma compartment within a few hours after i.v. administration. Assuming that the pattern of biliary excretion of cis-platinum or its metabolites in man is the same as that
observed in animal studies the second peak at 3.5 hr in our patient may be explained. This peak is the visual representation of the process of second influx. Sampling scheme, velocities of biliary excretion and reabsorption will determine the presence or absence of this peak in other patients.

Definite confirmation of a renewed influx of platinum in the plasma compartment due to absorption of cis-platinum excreted in the bile can only be done through bile cannulation and assay of platinum in the bile.

ACKNOWLEDGEMENTS

We wish to thank Bristol-Myers Company for kindly supplying purified cis-platinum for the assay standards.

REFERENCES


ERYTHROCYTE POLYAMINE LEVELS IN RATS WITH H4II-E HEPATOMAS BEFORE AND AFTER RADIATION TREATMENT

James R. Shipe, John Savory, Michael R. Wills, Roy Rowley, William B. Looney and Harold A. Hopkins

Depts. of Pathology, Biochemistry and Medicine (J.R.S, J.S., M.R.W.) Division of Radiobiology and Biophysics (R.R., W.B.L., H.A.H.) University of Virginia Medical Center Charlottesville, Virginia 22908

ABSTRACT

The concentration of the polyamines, spermidine and spermine associated with the erythrocytes of rats with H-4-II-E hepatomas increased with tumor growth. Following radiation therapy the erythrocyte spermidine and spermine levels decreased by 63 and 47% respectively. Six days after radiation treatment the erythrocyte polyamines had increased to the pre-treatment elevated levels. These data suggest that erythrocyte polyamine levels may be useful in assessing the response to therapy and in detecting the continued growth of the tumor in patients with malignant disease.

INTRODUCTION

Increases in urine and plasma polyamine concentrations have been demonstrated in patients with a variety of malignant diseases (Bachrach, 1976; Jänne, Pösö and Raina, 1978). The value, however, of plasma and urine polyamine measurements both for the specific diagnosis of malignant disease and as an early marker of malignancy appears to be limited (Cohen, 1977; Russell, 1977) due to the lack of sensitivity at early
stages of the disease and the relatively low specificity.

There are few studies on the concentration of polyamines in erythrocytes despite the fact that over 88% of the spermidine and 53% of spermine in the circulation are bound to these cells (Cooper, Shukla and Rennert, 1976). We have evidence that exogenous polyamines bind strongly to erythrocytes in vitro and that the endogenous erythrocyte polyamine concentration is not changed following acidic hydrolysis at elevated temperatures (Shipe and Savory, unpublished results). This is in contrast to other physiological fluids, in which the polyamines are in a conjugated form, possibly as the N-acetyl derivatives (Abdel-Monem, et al., 1978; Russell and Russell, 1975).

This association between free polyamines and erythrocytes has led to our hypothesis that in the circulation the erythrocytes may serve as passive "carriers" of the polyamines from sites of production to sites of conjugation and excretion.

Preliminary clinical studies (Savory, Shipe and Wills, 1979) on a small series of cancer patients prior to any modality of therapy demonstrated that erythrocyte polyamines were increased much more frequently than the corresponding plasma concentrations. Both plasma and erythrocyte polyamine concentrations were measured in a patient with breast cancer. Serial blood specimens were obtained prior to and during therapy with 5-fluorouracil. In the erythrocytes there was an immediate and dramatic fall in the elevated baseline spermidine concentration to slightly below normal limits within seven days of initiation of therapy. In contrast, the plasma concentrations of putrescine and spermidine increased immediately following the beginning of treatment and then
dropped towards the normal range by seven days. Spermine in both the plasma and erythrocytes remained unchanged. This data suggested erythrocyte polyamine analysis could be of value in monitoring the efficacy of therapy and the detection of recurrence of the tumor.

In the present report this theory was investigated in an animal model system. Hepatoma H-4-II-E is a fast growing solid tumor that readily metastasises to the lungs and regional lymph nodes. Radiation and chemotherapeutic studies show it to be resistant to therapy (Looney, Hopkins and Trefil, 1977) and as such a valuable model for establishing the principles of effective tumor management. We studied the correlation between tumor mass and erythrocyte polyamine levels in rats bearing H-4-II-E transplantable tumors. Additionally, we measured erythrocyte polyamine levels at various times following radiation therapy.

**MATERIALS AND METHODS**

**Reagents**

Putrescine, spermidine and spermine were obtained as their hydrochloride salts from Calbiochem-Behring Corp. (San Diego, CA 92112). Cadaverine and the internal standard, 3,3'-iminobispropylamine, were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI 53233).

The reagents used in buffer preparation were sodium citrate (dihydrate), sodium chloride, sodium hydroxide, disodium ethylenediaminetetraacetate, hydrochloric acid, and were all A.C.S. certified grade (Fisher Scientific Co., Fair Lawn, NJ 07410). The 30% aqueous solution of Brij 35 was obtained from Harleco (Gibbstown, NJ 08027).

Boric acid and potassium hydroxide (ACS certified, Fisher Scientific)
and 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY 14650) were used in the preparation of the o-phthalaldehyde reagent. The o-phthalaldehyde obtained from several sources gave comparable sensitivity; however, one source, "Fluoropa", (Pierce Chemical Co., Rockford, IL 61105), was found to dissolve more readily and exhibited a somewhat slightly decreased background fluorescence.

**Buffers**

Glass-distilled water was used in the preparation of the buffer system and their pH was adjusted with AR grade concentrated hydrochloric acid. Buffer 1: 0.067M sodium citrate·2H₂O (final Na⁺ concentration 0.20M), pH 5.80 ± 0.02. Buffer 2: 0.30M sodium citrate·2H₂O and 1.5M sodium chloride (final sodium concentration 2.40M, pH 4.68 ± 0.02. Buffer 3: 0.30M sodium citrate·2H₂O and 2.50M sodium chloride (final Na⁺ concentration 3.40M), pH 4.68 ± 0.02.

One ml of liquified phenol, approximately 88% (Mallinckrodt, Inc., St. Louis, MO) and 60 ml absolute ethanol were added per liter of each buffer. The o-phthalaldehyde fluorescent reagent, "Fluoropa", was prepared according to the manufacturer's instructions. The buffers and fluorescent reagent were filtered through a 0.22 μm membrane filter and de-gassed before placing in the liquid chromatograph.

Blood samples (4 to 6 ml) were collected from normal and tumor-bearing rats by cardiac puncture. Following centrifugation the plasma and buffy coat was aspirated and discarded. The packed erythrocytes were resuspended in an equal volume of normal saline and counted using a Model S Coulter automated cell counter. To 1.0 ml aliquots of the
suspended erythrocytes was added 3,3'-iminobispropylamine as an internal standard; the erythrocytes were lysed with a drop of Triton X-100 and proteins were precipitated with 2.0 ml of cold 10% trichloroacetic acid. The protein-free filtrates were taken to dryness under reduced pressure prior to chromatographic analysis.

An Aminco Aminalyzer (American Instrument Co., Silver Spring, MD 20910) high pressure liquid chromatograph fitted with a fluorometric detection system was used for the estimation of polyamines in the erythrocyte extracts. The system used an Aminco PA cation-exchange resin packed to a height of 6.0 cm in a 3.0 mm ID glass column. The polyamines were detected fluorometrically following the formation of o-phthalaldehyde derivatives.

The erythrocyte polyamines were separated in 30 min. with the 3-buffer system. Buffer 1 was pumped for 6 min. to elute the amino acids; buffer 2 was pumped for 12 min. to elute the internal standard and spermidine, and finally buffer 3 was pumped for 12 min. to elute spermine.

The buffer flow rate was 26 ml per hour and the o-phthalaldehyde reagent at 32 ml per hour. Following the analysis, the column is automatically regenerated by pumping a solution of sodium hydroxide (0.2M) and 0.67 mM disodium EDTA for 6 min. and re-equilibrating with the starting buffer for 18 minutes. Full details of the procedure will be published elsewhere (Shipe and Savory, 1980).

Tumor Transplantation

Hepatoma H-4-II-E cells were maintained in vitro in Swim's 77 medium with 25% serum (20% horse, 5% fetal bovine, GIBCO, NY). Cells
were passed weekly on attaining confluence. In vitro growth characteristics have been detailed earlier (Kovacs, Evans and Hopkins, 1977).

Subcutaneous inoculation of 2 x 10⁶ log phase cells in 0.1 ml serum free medium into the flank of male ACI rats (Laboratory Supply Company, Indianapolis, IN) gave rise to palpable tumors in 7 to 9 days. Rat weight at inoculation was 120 to 140 g. Animals were caged individually in an air-conditioned room lighted from 8 A.M. to 8 P.M. Rat chow and water were provided ad libitum.

Tumor volumes were calculated from measurements of length, width and height assuming that tumors were hemi-ellipsoids where volume = \( \frac{4\pi}{3} \times \frac{1}{2} \times W/2 \times H/2 \). This formula reduces to \( \frac{1}{2} \times LWH \). Treatments were given for volume regrowth experiments when volumes first exceeded 200 mm³.

X-rays for tumor irradiation were produced by a General Electric Maximar III 250 using a 250 kV accelerating voltage and a 15 mA filament current. The beam was filtered with 0.25 mm copper and 1.0 mm aluminum. Animals were ether anesthetized prior to irradiation and placed in a lead-shielded box through which the tumor was protruded. The midpoint of the tumor was approximately 6 cm from the x-ray tube target and received the dose indicated, while the animal body received 0.5% of that dose.

The machine output was routinely calibrated with a chamber type electrostatic probe (Victoreen, R-Meter). Radiation doses are given here as the machine output in roentgens (R).
Figure 1: Fluorescent detection of the polyanilines; putrescine (Put) spermidine (Spd) and spermine (Spm) in normal and tumor-bearing rats. (A) Tritiated propylamine (1) was added as an internal standard as described in Materials and Methods.

Figure 2: The effect of 1500 R日趋 ray on the growth of hepatoma H413E. Each point represents the mean ± S.E. for separate rats. □ radiation-treated tumor rats; ○ non-radiated control tumor rats.
RESULTS

Content of Putrescine, Spermidine and Spermine in Rat Erythrocytes

The concentration of the erythrocyte polyamines in ten normal adult rats revealed a mean spermidine concentration of 1.6 nmoles/10^9 cells (range 1.36 - 1.79 nmoles/10^9 cells) which approximates that found in human erythrocytes (Shipe and Savory, 1980). The mean spermine concentration in these rats (0.5 nmoles/10^9 cells; range 0.30-0.72 nmoles/10^9 cells) was approximately 40 percent less than that found in human erythrocytes. In both normal and tumor-bearing rats the erythrocyte putrescine concentration is quite small and therefore was not quantitated routinely. Figure 1 shows the chromatograms from the analysis of erythrocytes from both a normal and a tumor-bearing rat.

Erythrocyte Polyamines During Tumor Growth

Changes in tumor volume growth following exposure to 1500 R x-rays are given in Figure 2. Untreated tumors have a volume doubling time to 1400 mm^3 of 49.2 hrs., reaching a volume of 20,000 mm^3 in approximately 20 days. Retarded growth due to irradiation is evident by one day following treatment up to day 7, thereafter tumors resume growth at a rate equivalent to controls. The erythrocyte spermidine and spermine concentration was measured in six untreated tumor-bearing rats and three normal controls. With only a single exception, it was found that the erythrocyte spermidine concentration increased with increasing tumor mass (Figure 3). There was no significant change in the erythrocyte spermine concentration in the tumor-bearing rats as compared to the normal controls.
Figure 3: Changes in the level of erythrocyte spermidine in rats bearing H4IIE hepatomas at various stages of tumor growth.

Several rats were not sacrificed until the tumor mass had reached the 6 to 12 gm range. As with the smaller size tumors, the increase in erythrocyte spermidine paralleled the increase in tumor mass. After the individual tumors exceeded 10 gms the concentration of erythrocyte spermidine appeared to approach a saturation level (12 nmoles per 10^9 erythrocytes) of almost 10 times the normal mean value. The erythrocyte spermine concentration was increased 2 to 3 times the normal mean level; however, there was no correlation between the tumor size and the spermine concentration. The important finding in this preliminary study was that in all of the 24 untreated tumor-bearing rats studied, there was a significant increase in the erythrocyte spermidine concentration and, although the absolute concentration varied with tumor size, there was no overlap with the range of the normal controls.
Effect of Radiation on Erythrocyte Polyamines

A single treatment with 1500 rads was given to 6 tumor-bearing rats, 3 of which were sacrificed approximately one hour later. Following irradiation of the tumors the erythrocyte spermidine had decreased an average of 62% (range 0.86-1.23 nmol/10⁹ cells) when compared to the levels observed in untreated rats having similar sized tumors (range 2.62-3.45 nmol/10⁹ cells). The decrease in erythrocyte spermine was approximately 47% (range 0.15-0.23 nmol/10⁹ cells) of the pre-irradiation level. The tumor-bearing rats which were sacrificed 6 days after radiation therapy had erythrocyte spermidine and spermine levels which were identical to those obtained from non-irradiated controls with similar sized tumors.

DISCUSSION

The use of polyamines as biological markers of tumor growth and regression has been studied in several in vitro tumor cell systems (Andersson and Heby, 1972; Heby et al, 1975; Pohjanpelto, 1973; Woo and Simon, 1973). Extracellular polyamines in serum have been investigated in experimental tumor systems as a marker of tumor cell kill in response to therapy (Russell et al, 1974; Russell et al, 1976; Russell et al, 1974).

In the present report, the concentration of erythrocyte polyamines was examined in rats bearing H-4-II-E hepatomas. Early in the course of tumor growth there are profound elevations in the erythrocyte spermidine. For example, Figure 1 (B) shows the nearly two-fold increase in erythrocyte spermidine observed in a rat having a tumor volume of 900 mm³ (1.10 gm wet weight). The erythrocyte spermine concentration was not significantly elevated as compared to normals. As mentioned earlier, the erythrocyte putrescine concentration in both normal and
tumor-bearing rats was considerably lower than the spermidine and spermine content and, although measurable, was not quantitated.

The erythrocyte spermidine concentration exhibited a significant positive correlation with the tumor mass. The increases in erythrocyte spermidine appear to accurately reflect small increases in tumor mass (Figure 3). Although erythrocyte spermine concentrations were elevated in rats bearing very large tumors, there was no significant correlation with tumor mass (data not shown). The relationship between erythrocyte spermidine and tumor mass is more accurately described in terms of the growth rate of the tumor. The highest erythrocyte spermidine concentration per gram of tumor is observed during the intervals of highest specific growth rate. The growth rate of hepatoma H-4-II-E decreases with increasing tumor volume (Figure 2). For tumors of less than 1000 mm$^3$ the volume doubling time (Td) was about 2 days. After the mean tumor exceeded 1000 mm$^3$, the volume doubling time decreased to about 4.0 days. The rapidly declining growth rate of large tumors is responsible for the apparent saturation of erythrocytes with spermidine. Our in vitro binding studies have demonstrated the ability of erythrocytes to bind 3 to 4 times this amount of spermidine, so the phenomenon probably represents decreased production rather than lack of available erythrocyte binding sites.

The response of the rat hepatoma H-4-II-E to a single dose of x-radiation is shown in Figure 2. A 1500 R dose results in rapid tumor regression. The tumor growth slowed within 24 hours of exposure, however, resumed normal growth rate within 6 to 7 days after radiation. In previous studies it was clearly demonstrated that the apparent changes in tumor volume which are only observed 24 hours following x-radiation
do not reliably reflect the initial response of its constituent cells. For example, $^3$H-thymidine incorporation into tumor DNA is markedly depressed within one hour after irradiation (Kovacs et al, 1976). Similarly, in this study we observed a dramatic decrease in the erythrocyte spermidine and spermine one hour following irradiation as compared to non-irradiated controls. Similar doses of 1500 rads given to normal controls did not reduce the erythrocyte polyamine levels, suggesting that the immediate decrease seen in tumor-bearing rats was the result of decreased production by the tumor followed by a rapid clearance of the polyamines from the circulation and not a systemic effect of radiation per se. In a previous study we found that within 10 minutes following I.V. injection of $^{14}$C polyamines, erythrocyte spermidine and spermine had decreased by 80 and 70% respectively (Wills, Shipe and Savory, 1980). Six days following radiation the tumor growth rate and erythrocyte spermidine and spermine had returned to levels identical to the non-irradiated controls.

On the basis of these animal studies and preliminary clinical studies on a number of cancer patients (Savory, Shipe and Wills, 1979) it appears that erythrocyte spermidine may be a useful marker of the efficacy of various modalities of therapy and aid in the detection of recurrent tumor growth.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of Dr. Glenys S. Rees in this study.
REFERENCES


Savory, J., Shipe, J.R. and Wills, M.R.: Erythrocyte polyamines as biological markers in patients with malignant disease. (Submitted for publication)


Copyright © 1980 By
PJD Publications Ltd., Box 966, Westbury, N.Y., 11590
THE EFFECT OF ANTIDEPRESSANTS ON L-5HTP-INDUCED CHANGES
IN RAT PLASMA CORTICOSTEROIDS

Robert A. Lahti and Craig Barsuhn

CNS Diseases Research
The Upjohn Company
Kalamazoo, Michigan 49001

ABSTRACT

The finding that L-5HTP will cause an elevation in rat plasma corticosteroids, presumably through generation of serotonin, has been utilized to evaluate the serotonin potentiating and antagonizing properties of a series of antidepressants. Fluoxetine and zimelidine were most effective in enhancing the effect of low dose (12.5 mg/kg) L-5HTP. Agents such as chlorimipramine, viloxazine and opipramol were weakly active at potentiating L-5HTP and mianserin and dibenzepine were strong antagonists of the L-5HTP effect. Since antidepressants have been found to either potentiate or antagonize serotonin, it is difficult to envision that the antidepressants exert their therapeutic effect through serotonergic mechanisms.

INTRODUCTION

Pharmacological studies (Sigg et al., 1963) have demonstrated that antidepressants potentiate the effects of serotonin as well as, or better than, they potentiate the effects of the catecholamine, norepinephrine. Evidence has recently been presented showing that L-5HTP treatment can elevate rat plasma corticosteroid levels and do so presumably through its decarboxylated product, serotonin (Fuller et al., 1975). They also presented evidence showing that the specific serotonin uptake blocker, fluoxetine, could enhance the elevation of corticosteroid levels produced by L-5HTP. In light of the above evidence, it seemed reasonable that antidepressants may also potentiate the corticosteroid elevating effect of
L-5HTP.

METHODS

Male Upjohn Sprague-Dawley rats weighing approximately 240 grams were housed in groups of 5 for several days prior to each experiment. On the day of the experiment, the rats were injected at zero hour with drug or vehicle and 1 hour later they were given an i.p. injection of L-5HTP. One hour after the L-5HTP injection, the animals were anesthetized with halothane and 4-5 ml of blood withdrawn via heart puncture with a heparinized syringe. Plasma corticosteroids were determined as previously described (Lahti and Barsuhn, 1974).

RESULTS

When rats were pretreated with fluoxetine and then given 12.5 mg/kg L-5HTP, we observed, as reported by others (Fuller et al., 1975) that there was an enhancement of the effect of L-5HTP on plasma corticosteroids (Table 1). A series of compounds, mainly antidepressants, was then tested for their ability to enhance the effect of 12.5 mg/kg L-5HTP. Of many compounds tested, only fluoxetine and zimelidine showed a statistically significant enhancement of the L-5HTP effect. Other compounds did enhance the effect, however the results were not statistically significant. Of considerable interest was the finding that 10 mg/kg chlorimipramine, a strong serotonin uptake blocker, did cause an enhancement, although not a statistically significant one. Two of the antidepressants tested, mianserin and dibenzepine, as well as the serotonin antagonist, cyproheptadine, antagonized the very moderate corticosteroid
elevating effect of 12.5 mg/kg of L-5HTP. The antagonistic effect of the above two antidepressants is probably a reflection of the serotonin antagonistic effect that some antidepressants exhibit at high doses (van Dorsser and Dresse, 1976).

To more effectively evaluate the serotonin antagonistic activity of this series of compounds, a dose of 25 mg/kg of L-5HTP was administered so that a large increase in corticosteroids was elicited. The results of this series of experiments are also presented in Table 1. Several compounds at doses of 10 mg/kg i.p. were found to significantly inhibit the corticosteroid elevating effect of a 25 mg/kg dose of L-5HTP. Fluoxetine, zimelidine and chlorimipramine did not cause a significant reduction, nor did viloxazine, opipramol or amitriptyline.

Testing of additional compounds showed that diazepam and diphenhydramine were both ineffective as either enhancers or antagonists of the L-5HTP effect. Nefopam, a non-narcotic analgesic which had previously been evaluated as an antidepressant (Bassett et al., 1969), was also tested in this procedure and found to be inactive.

DISCUSSION

The finding by others (Fuller et al., 1975) that L-5HTP will cause an elevation in rat plasma corticosteroids has been utilized to evaluate the serotonin potentiating and antagonizing properties of a series of compounds. When relatively small doses of L-5HTP were used, an enhancement of the effects was produced by the serotonin uptake blockers, fluoxetine and zimelidine.
Table 1

Effect of Antidepressants on L-5HTP Induced Elevations in Rat Corticosteroids

<table>
<thead>
<tr>
<th>% 12.5 mg/kg 5HTP</th>
<th>Compound</th>
<th>% 25 mg/kg 5HTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>830*</td>
<td>Fluoxetine</td>
<td>78</td>
</tr>
<tr>
<td>704*</td>
<td>Zimelidine</td>
<td>80</td>
</tr>
<tr>
<td>347</td>
<td>Chlorimipramine</td>
<td>69</td>
</tr>
<tr>
<td>287</td>
<td>Viloxazine</td>
<td>100</td>
</tr>
<tr>
<td>206</td>
<td>Opipramol</td>
<td>71</td>
</tr>
<tr>
<td>167</td>
<td>Imipramine</td>
<td>47*</td>
</tr>
<tr>
<td>155</td>
<td>Protriptyline</td>
<td>52*</td>
</tr>
<tr>
<td>100</td>
<td>Amitriptyline</td>
<td>68</td>
</tr>
<tr>
<td>80</td>
<td>Trimipramine</td>
<td>42*</td>
</tr>
<tr>
<td>25*</td>
<td>Dibenzepine</td>
<td>38*</td>
</tr>
<tr>
<td>38*</td>
<td>Mianserin</td>
<td>21*</td>
</tr>
<tr>
<td>92</td>
<td>Diazepam</td>
<td>NT</td>
</tr>
<tr>
<td>138</td>
<td>Diphenhydramine</td>
<td>112</td>
</tr>
<tr>
<td>82</td>
<td>Nefopam</td>
<td>76</td>
</tr>
<tr>
<td>26*</td>
<td>Cyproheptadine</td>
<td>58</td>
</tr>
</tbody>
</table>

*Significantly different from L-5HTP treatment at p = 0.05. NT = Not Tested.

All compounds were tested at 10 mg/kg i.p.

<table>
<thead>
<tr>
<th>Drug</th>
<th>L-5HTP</th>
<th>Bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Hr</td>
<td>1 Hr</td>
<td>2 Hr</td>
</tr>
</tbody>
</table>
The effect of chlorimipramine was not as large as expected considering its serotonin uptake blocking activity. This reduced effect of chlorimipramine could be accounted for by assuming the compound also has serotonin antagonistic activity. The two effects oppose each other and the net result is explained. When larger doses of L-5HTP were used, 5 of 9 antidepressants tested were found to significantly reduce the corticosteroid elevating property of L-5HTP. It is possible that the effects of high and low dose L-5HTP alter plasma corticosteroids by different mechanisms, as has been shown for the benzodiazepines (Lahti and Barsuhn, 1975), thus accounting for the apparent agonist-antagonist effect of certain drugs.

The activity of this series of compounds can be explained by the same arguments that were used to explain chlorimipramine activity. Certain compounds, such as fluoxetine and zimelidine, and possible viloxazine, are potentiators of serotonin with little or no antagonistic activity. Chlorimipramine potentiates low doses of 5-HTP and slightly antagonizes high doses, indicating it has more antagonistic activity than the aforementioned compounds. Trimipramine, imipramine and protriptyline are definitely strong antagonists of serotonin with little potentiating effect. Finally, mianserin and dibenzepine can be categorized as simply strong antagonists. This interpretation is diagrammed in Table 2.

Since the antidepressants exert such diverse effects on serotonin in this procedure, no common theme appears. Consequently, it is difficult to envision that their mode of action is through serotonin.
Table 2
Antidepressants Ranked According to Serotonin Antagonistic and Potentiating Properties

<table>
<thead>
<tr>
<th>Str</th>
<th>Fluoxetine</th>
<th>Zimelidine</th>
<th>Viloxazine</th>
<th>Imipramine</th>
<th>Protriptyline</th>
<th>Trimipramine</th>
<th>Mianserin</th>
<th>Dibenzepine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS
To Eli Lilly, Astra, Ciba-Geigy, Stuart, Merck, Sharp and Dohme, Ives, Sandoz, Organon, Hoffmann-LaRoche, and Riker pharmaceutical companies for generously supplying samples of their respective compounds.

REFERENCES


DIESTER METABOLITES OF THE FLAME RETARDANT CHEMICALS, TRIS(1,3-DICHLORO-2-PROPYL)PHOSPHATE AND TRIS(2,3-DIBROMOPROPYL)PHOSPHATE IN THE RAT: IDENTIFICATION AND QUANTIFICATION

R. K. Lynn, K. Wong, R. G. Dickinson, N. Gerber and J. M. Kennish
Department of Pharmacology, Medical School
University of Oregon Health Sciences Center
Portland, Oregon

ABSTRACT

Radiolabeled tris(1,3-dichloro-2-propyl)phosphate (TDCP), and tris(2,3-dibromopropyl)phosphate (TDBP), were administered intravenously to male Sprague Dawley rats. After 120 hr, 53.99 ± 3.14 and 57.27 ± 4.37 percent (mean ± S.D.) of the administered radiolabel had been excreted in the urine of TDCP and TDBP treated rats, respectively. The major urinary metabolite of TDCP was identified as bis(1,3-dichloro-2-propyl)phosphate by gas chromatographic-electron impact-mass spectrometric (GCMS) analysis of its trimethylsilyl derivative. The mass spectrum displayed several rearrangement ions including apparent single (m/e 155, base peak; m/e 265) and double (m/e 281) hydrogen atom transfers. The structure of the diester metabolite of TDCP was confirmed by GCMS comparison with the chemically synthesized material. The structure of the diester metabolite of TDBP, bis(2,3-dibromopropyl) phosphate, was determined by mass spectrometric analysis (direct probe) of its methyl derivative. The mass spectrum displayed ions resulting from fragmentation of the halo-alkyl group(s) and from apparent hydrogen atom rearrangement. High performance liquid chromatographic analysis of the urine (0-120 hr) demonstrated that the diester metabolites of TDCP and TDBP accounted for 62.3 ± 8.3 and 7.8 ± 1.2 percent (mean ± S.D.) of the radiolabel present in urine, respectively.

INTRODUCTION

Tris(1,3-dichloro-2-propyl)phosphate (TDCP), and tris(2,3-dibromopropyl)phosphate (TDBP), are flame retardant chemicals (Fig. 1) formerly used in the apparel industry to confer flame-resistant properties to
polyester fabric (Saunders, 1978). Of special relevance was the use of
TDCP and TDBP in children's sleepwear which is required by law to meet
flame resistance standards. In 1977 TDBP was banned for use in sleep-
wear due to its carcinogenicity in rats and mice (National Cancer Insti-
tute, 1978; Van Duuren et al.,1978). TDCP which was temporarily used as
a substitute for TDBP in sleepwear is undergoing a two-year bioassay
sponsored by the Stauffer Chemical Co. Currently, the major use of TDCP
is as a flame retardant in flexible polyurethane foam.

TDCP (Gold et al., 1978) and TDBP (Prival et al., 1977; Blum and
Ames, 1977) are mutagens in Salmonella-mammalian liver homogenate tests.
In the absence of liver microsomes, TDCP displayed no mutagenic activity
and the mutagenicity of TDBP was markedly decreased. Therefore, bio-
transformation products of TDBP and TDCP are the suspected mutagenic
and potentially carcinogenic (McCann and Ames, 1976) agents. 2,3-Dibro-
mopropanol, a metabolite of TDBP in rats (St. John, 1976) and man (Blum
et al., 1978) is less mutagenic than TDBP (Prival et al., 1977;
Blum and Ames, 1977). Gold et al. (1978) have proposed two hypothetical
metabolites of TDCP, 1,3-dichloro-2-propanol and 1,3-dichloro-2-propanone and shown that these chemicals are in fact mutagenic in the Salmonella tests. However, the actual metabolic fate of TDCP has not been reported. In the present study, using radiotracer techniques and GCMS analysis, we have identified the major metabolite of TDCP and a minor metabolite of TDBP in rat urine.

MATERIALS AND METHODS

Materials. TDCP was a gift from Stauffer Chemical Co. TDBP was obtained from K and K Laboratories (Plainview, NY). Other chemicals and suppliers were as follows: diethyl ether (analytical reagent), Mallinkrodt Chemical Works (St. Louis, MO); pyridine, Supelco (Bellefonte, Penn.); N,N-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), Pierce Chemical Co. (Rockford, Ill.); Emulphor 620 (GAF Corp., Linden, NJ); 14C-TDCP (2-propyl-1,3-14C, 14C-TDBP(1-propyl-1-14C) and Aquasol (New England Nuclear, Boston, Mass.). Ethereal diazomethane was prepared from N-methyl-N'-nitro-N-nitroso- guanidine (Aldrich Chemical Co., Milwaukee, Wis.). Male Sprague Dawley rats were obtained from King Laboratories (Oregon, Wis.).

Synthesis of Bis(1,3-dichloro-2-propyl)phosphate. TDCP (100 mg; 10 μCi/100 mg) in ethanol (100 μl) was added to a mixture of acetone (4 ml), water (2.5 ml) and sodium hydroxide (150 μl, 12 N). After five hours the reaction mixture was titrated to pH 7.6 by the addition of phosphoric acid and phosphate buffer (pH 8.0, 0.5M). The solution was evaporated to dryness under vacuum and redissolved in water (10 ml). The aqueous phase was extracted twice with diethyl ether (10 ml) and the organic phases discarded. The aqueous phase was titrated to pH 1 with hydrochloric acid (12 N) and extracted with ether (10 ml x 2). High performance liquid chromatographic (HPLC) analysis of the combined ether extracts indicated that the radiolabeled product was greater than 95% pure. GCMS analysis of the product following derivatization with BSTFA (1% TMCS) in pyridine confirmed that the product was bis(1,3-dichloro-2-propyl)phosphate.

TDCP: Metabolite Extraction and Derivatization. 14C-TDCP (80 mg; sp. ac. 10 μCi/100 mg) dissolved in Emulphor 620 (8 ml) was administered intraperitoneally to a male Sprague Dawley rat. The animal was housed in a glass metabolism cage which permitted the separate collection of urine and feces. Urine was cooled in an ice bath during the collection period and stored at -20°C prior to analysis. Two ml of the first 24 hr urine sample was adjusted to pH 8.5 with phosphate buffer (0.5 ml; pH 8.5; 0.5M) and extracted with diethyl ether (10 ml x 2). The ether layers were discarded and the lower layer titrated to pH 1 with 12N HCl. The aqueous layer was extracted with diethyl ether (10 ml) and 2 ml of the organic phase evaporated to dryness under nitrogen. The residue was
dissolved in pyridine (50 microliters) and BSTFA (30 microliters) containing 1 percent TMCS was added. Urine obtained from a control animal after intraperitoneal administration of Emulphor 620 was extracted and derivatized similarly.

TDBP: Metabolite Extraction and Derivatization. $^{14}$C-TDBP (25 mg; sp.ac. 65.2 $\mu$Ci/100 mg) dissolved in Emulphor 620 was administered intraperitoneally to a male Sprague Dawley rat. Urine was collected and stored as described above. The 24 hr urine sample was analyzed by HPLC and the fraction eluting between 15 and 17 min combined and evaporated to dryness. The residue was redissolved in methanol and treated with ethereal diazomethane. After 24 hr the solvents were evaporated and the residue transferred in methanol to a glass, SE-30 coated (Thenot, 1979), direct probe vial. The methanol was evaporated by impingement with nitrogen.

Metabolite Quantification. TDCP (342 mg, 10 $\mu$Ci) and TDBP (1.76 mg, 10 $\mu$Ci) were administered intravenously to rats in 25% Emulphor 620. Each flame retardant was studied in four rats. Urine was collected for five days at 24 hr intervals as described above and aliquots (0.1 ml) analyzed by liquid scintillation counting in Aquasol (10 ml). The diester metabolites were quantified by direct HPLC analysis of a composite (0-120 hr) urine sample.

Chromatography. The derivatized urine extracts were analyzed on a Hewlett Packard model 5830 gas chromatograph equipped with a flame ionization detector and a 1.5 m x 2 mm i.d. glass column packed with 1.5% OV-101 on Gas-Chrom Q (100-120 mesh). The column temperature was maintained at 100°C for 1 min then programmed to 320°C at 10°C per min. Hydrogen, air and nitrogen flows were 30, 250, and 30 ml/min respectively.

Urine containing radiolabeled metabolites was analyzed directly on a Hewlett Packard model 1084A liquid chromatograph equipped with a 25 cm x 4.6 mm i.d. column packed with RP-8. Analyses were performed at room temperature by gradient elution with acetonitrile and aqueous hydrochloric acid (pH 2.3). The percent acetonitrile was increased in a linear fashion from 5% to 95% over a period of 30 min. Total flow was maintained at 2 ml/min. The column effluent was collected in 1 ml fractions and analyzed by liquid scintillation counting with 10 ml Aquasol in a Beckman model LS-330. Under these conditions the diester metabolites of TDCP and TDBP had retention times of approximately 15-17 min.

Mass Spectrometry. Mass spectra were obtained in a Finnigan model 4021 gas chromatograph-mass spectrometer-data system equipped with a jet separator and a dual electron impact-chemical ionization source. Spectra were obtained in the electron impact mode at 280°C with an ionizing potential of 70eV. Chromatographic conditions were as described above using helium (25 ml/min) as the carrier gas. The direct probe was maintained at 100-150°C.
RESULTS

TDCP. Gas chromatograms of derivatized extracts from control urine and urine from the TDCP treated rat are illustrated in Figure 2. Urine from the TDCP treated rat contained one major component which was not present in control urine. This component was identified by mass spectrometry (Fig. 3) as the mono-trimethylsilyl (Tms) derivative of bis(1,3-dichloro-2-propyl)phosphate. No molecular ion was observed. Fragment ions at m/e 375, 355, and 341 were produced by loss of CH$_3$, Cl, and CH$_2$Cl from the molecular ion, respectively. Cleavage of the oxygen-carbon bond accompanied by double hydrogen rearrangement produced the isotope cluster at m/e 281. Loss of both dichloroisopropyl substituents, a methyl group from Tms, and hydrogen rearrangement produced the base peak at m/e 155. Similar GCMS analysis of the Tms derivative of chemically synthesized bis-(1,3-dichloro-2-propyl)phosphate produced a mass spectrum identical to that shown in Figure 3.

After 5 days 53.99 ± 3.14 percent (mean ± S.D.) of the intravenously administered radiolabel had been excreted in the urine. The radio-labeled components present in the composite (0-120 hr) urine samples were separated by HPLC. The major component, which accounted for 62.3 ± 8.3 percent (mean ± S.D.) of the radiolabel in urine was identified by GCMS analysis of its Tms derivative as bis(1,3-dichloro-2-propyl)phosphate.

TDBP. Rats excreted 57.21 ± 4.37 percent (mean ± S.D.) of the intravenously administered radiolabel in the urine (0-120 hr). HPLC analysis of the composite (0-120 hr) samples demonstrated that the radiolabeled component eluting at 15-17 min accounted for 7.8 ± 1.2 percent (mean ± S.D.) of the radiolabel in urine. This component was identified by mass spectro-
Fig. 2

Gas chromatograms of control urine and urine from a rat treated with tris(1,3-dichloro-2-propyl)phosphate (TDCP).
Fig. 3. Electron impact mass spectrum of the trimethylsilyl (Tms) derivative of bis(1,3-dichloro-2-propyl)phosphate. Numbers in parentheses indicate the number of chlorine atoms present in the decomposition ions.

Fig. 4. Electron impact mass spectrum of the methyl derivative of bis-(2,3-dibromopropyl)phosphate. Numbers in parentheses indicate the number of bromine atoms present in the decomposition ions.
metric analysis of its methyl derivative as bis(2,3-dibromopropyl)phosphate. The mass spectrum (Fig. 4) contained ions produced by loss of Br and/or HBr from the halo-alkyl substituents. Other ions produced by loss of the halo-alkyl group(s) accompanied by single and double hydrogen atom rearrangements were also observed. No molecular ion was observed.

**DISCUSSION**

The data presented in this report unequivocally identify the major urinary metabolite of TDCP in the rat as bis(1,3-dichloro-2-propyl) phosphate. The corresponding diester metabolite of TDBP, bis(2,3-dibromopropyl)phosphate was also identified in rat urine. However, it is apparent from these studies that the diester metabolite of TDBP represents only a minor urinary biotransformation product. Mass spectra of both the diester metabolites contained fragment ions which resulted from apparent double hydrogen rearrangements similar to those observed by McLafferty (1956), Quayle (1959) and Bafus (1966) for triethylphosphosphate. The apparent double hydrogen rearrangement also occurs in the electron impact mass spectra of TDCP and TDBP.

The ester bond of organophosphorous alkyl triesters is cleaved in mammals to produce organophosphorous diesters via several mechanisms: (1) mixed function oxidase reactions (2) hydrolase reactions and (3) glutathione S-alkyl transferase reactions (Dauterman, 1971). Identification of the diester metabolites of TDCP and TDBP does not, therefore, establish the mechanism of their production in as much as each of the above described reactions would produce the same diester. In addition to the diester metabolites of TDCP and TDBP several other radiolabeled components were observed in rat urine. In conjunction with *in vitro*
studies currently underway in this laboratory, the identification of these additional metabolites will assist in establishing the mechanism of cleavage of these phosphate ester bonds. Also, mutagenic testing (Gold et al., 1978) of the diester metabolites of TDCP and TDBP, as well as the other metabolites observed in urine may help to establish the identity of the actual mutagenic agents produced by the biotransformation of TDCP and TDBP.

ACKNOWLEDGEMENTS

This investigation was supported by NIEHS N01-ES-7-2126, DA 00942, NINCDS N01-NS-5-2328, and the Rose E. Tucker Charitable Trust.

REFERENCES


propanol. Sci. 201, 1020-23.


VITAMIN E AND THE INHIBITION OF PLATELET LIPOXYGENASE

Ephraim T. Gwebu, Ronald W. Trewyn, David G. Cornwell and Rao V. Panganamala

Department of Physiological Chemistry and the Comprehensive Cancer Center
The Ohio State University Columbus, Ohio 43210

ABSTRACT

Lipoxygenase was assayed by the formation of hydroxy-eicosatetraenoic acid (HETE) from arachidonic acid in human and rabbit washed platelets. Platelets from vitamin E-supplemented rabbits had much less lipoxygenase activity than platelets from either vitamin E-deficient or normal rabbits. Human and rabbit platelets preincubated with vitamin E had lowered lipoxygenase activity. These data show that vitamin E inhibits platelet lipoxygenase. Vitamin E and vitamin E acetate, in vitro, were equally effective inhibitors of lipoxygenase. Tween 20, in vitro, was a highly effective inhibitor of lipoxygenase. These data show that vitamin E functions, in vitro, as a surfactant in the inhibition of platelet lipoxygenase.
INTRODUCTION

Vitamin E is a highly effective inhibitor, in vitro, of a plant lipoxygenase (EC 1.13.11.12) isolated from soybeans (György and Tomarelli, 1944; Tappel et al., 1953; Panganamala et al., 1977). Several recent studies present conflicting data on the inhibition of a mammalian lipoxygenase such as platelet lipoxygenase with this antioxidant (Rao et al., 1978; Butler et al., 1979). It is important to establish whether vitamin E functions as an inhibitor of mammalian lipoxygenase. Polyunsaturated fatty acids are highly specific inhibitors of mammalian cell proliferation (Huttner et al., 1978; Cornwell et al., 1979). Vitamin E and other antioxidants overcome fatty acid inhibition and stimulate cell proliferation (Cornwell et al., 1979; Miller et al., 1980). If vitamin E inhibits mammalian lipoxygenase, the studies with polyunsaturated fatty acids and vitamin E suggest that cell proliferation is controlled in part by the oxidant stress supplied by hydroperoxy fatty acids which are products of the lipoxygenase reaction (Cornwell et al., 1979; Miller et al., 1980).

The oxidant stress hypothesis is consistent with many known effects of vitamin E and unsaturated fatty acids. Enhanced cell proliferation was one of the first biological effects identified with vitamin E (Mason, 1933). Other antioxidants such as butylated hydroxy toluene also stimulate cell proliferation (Witschi and Lock, 1972). Rapidly proliferating cells are resistant to peroxidation (Arneson et al., 1978). Lipid peroxides are reduced in tumor bearing animals and almost absent from the tumors themselves (Apffel, 1978).
In the present study, the activity of platelet lipoxygenase was measured by the conversion of arachidonic acid to 12-hydroxyeicosatetraenoic acid (HETE) in the presence of sufficient indomethacin to inhibit the cyclooxygenase pathway (Hamberg and Samuelsson, 1974; Nugteren, 1975; Ho et al., 1977; Dutilh et al., 1979). In some experiments, lipoxygenase activity was assayed in platelets obtained from animals maintained on vitamin E-deficient and vitamin E-supplemented dietary regimens. In other experiments, washed platelets were preincubated with vitamin E and lipoxygenase assayed. Finally, washed platelets were preincubated with either vitamin E acetate or Tween 20 in order to determine whether vitamin E acted as a radical scavenger or surfactant (Gutteridge, 1978; Mino and Sugita, 1978).

MATERIALS AND METHODS

Materials: d-α-Tocopherol (vitamin E), d-α-tocopheryl acetate (vitamin E acetate) and d-α-tocopheryl quinone (vitamin E quinone) were purchased from Eastman Organic Chemicals (Rochester, N.Y.) [1-14C] arachidonic acid (50 μCi/μmole) was purchased from New England Nuclear Co. (Boston, Mass.) The fatty acid was monitored for purity by thin layer chromatography (TLC) on a precoated plate (Whatman, LK6D) using ethyl acetate/2,2,4-trimethylpentane/acetic acid (100:100:1, v/v) as the developing solvent and a potassium iodide-starch spray. 5,8,11,14-eicosatetraynoic acid (TYA) was kindly supplied by Hoffman La-Roche (Nutley, N.J.) Indomethacin was kindly supplied by Merck, Sharp and Dohme Research Laboratories (Rahway, N.J.) Thromboxane B2 (TXB2) was kindly supplied by The Upjohn Co. (Kalamazoo, Mich.)
Preparation of Washed Platelets: Human platelet concentrates were kindly supplied within 72 h of their expiration date by the Blood Bank Laboratory of the University Hospital and the Regional Red Cross Blood Bank. Platelet rich plasma (PRP) was prepared from human platelet concentrate and from rabbit blood collected in plastic tubes containing sodium citrate (9 volumes of blood and 1 volume of 3.8% sodium citrate). The platelet concentrate or blood was centrifuged at 250 x g for 15 min to obtain PRP.

About 20 ml of PRP was centrifuged at 2,000 x g for 20 min. The platelet pellet was gently dispersed in 30 ml of a buffer containing 50 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA adjusted to pH 7.4. This suspension was centrifuged at 2,000 x g for 20 min and the washed platelet pellet was resuspended in 30 ml of the same buffer. Platelet protein was measured by a standard procedure (Lowry et al., 1951). The platelet count was determined by phase contrast microscopy.

Lipoxygenase Assay: Washed platelets were preincubated for 2 min with 0.3 mM indomethacin added in 5 μl ethanol. The antioxidant was dissolved in ethanol and a 5 μl aliquot was then added to 0.5 ml of the platelet suspension. Five μl of ethanol alone was added to the control sample. These platelet suspensions were mixed briefly on a Vortex mixer. The mixture was preincubated at 37°C for 5 min. Labeled arachidonic acid, 460 pmoles dissolved in 5 μl of ethanol, was then added to the mixture and mixed briefly. The incubation was continued for 15 min. The reaction was stopped by the addition of 150 μl of 1 N HCl. Unreacted fatty acid and HETE were extracted twice with 3 ml portions of ethyl acetate. Over 90% of the added label was extracted.
Ethyl acetate was removed on a Buchii evaporator and the residue was dissolved in 200 μl of methanol/ethyl acetate (1:1, v/v). An aliquot of this solution was placed on a precoated TLC plate (Whatman, LK6D) and the plate was developed with a solvent (Ho et al., 1977) containing ethyl acetate/2,2,4-trimethylpentane/acetic acid (100:100:1). Bands corresponding to arachidonic acid (Rf: 0.56) and HETE (Rf: 0.41) were scraped from the plate, mixed with 15 ml of Scintisol (Isolab, Akron, Ohio) and counted.

**Separation of Vitamin E and Its Derivatives:** Vitamin E, vitamin E acetate, and vitamin E quinone were separated by high performance liquid chromatography (HPLC) using a modification of a published procedure (Hatam and Kayden, 1979). Analyses were performed on a Model 322 MP chromatograph (Altex, Berkeley, Calif.) using a 30 cm X 4 mm id stainless steel tube pre-packed with Micropak MCH-10 (Varian, Palo Alto, Calif.) and methanol-water (94:6) as the mobile phase. The flow-rate was 2 ml/min. Peaks were detected with a Varichrom Variable wavelength UV detector (Varian) at the absorbance maximum for each component: 264 nm, vitamin E quinone; 284 nm, vitamin E acetate; 294 nm, vitamin E. Elution times were: 6 min, vitamin E quinone; 9.7 min, vitamin E; 13.2 min, vitamin E acetate.

**Dietary Regimens:** The vitamin E-deficient diet followed the formulation of Tappel and Zalkin (1959). The vitamin E-supplemented diet consisted of the vitamin E-deficient diet supplemented with 1 g of vitamin E acetate per kg of diet. These diets were formulated by ICN Pharmaceutical Co. (Cleveland, Ohio). Purina rabbit chow was used as the standard diet. Male 4 to 5 weeks old New Zealand white rabbits
were maintained on these diets until animals on the deficient diet developed symptoms of muscular dystrophy (4 to 5 weeks). Animals were housed in individual cages and the diets were fed, ad libidum.

RESULTS

Recovery of HETE and Arachidonic Acid from Washed Platelets: Platelets which are incubated with arachidonic acid produce HETE from the lipoxygenase pathway and both 12-hydroxyheptatrienoic acid (HHT) and thromboxane A$_2$ (TXA$_2$) from the cyclooxygenase pathway (Hamberg and Samuelsson, 1974; Nugteren, 1975; Ho et al., 1977; Dutilh et al., 1979). TXA$_2$ is unstable and readily converted to thromboxane B$_2$ (TXB$_2$). Products from the incubation of rabbit platelets with labeled arachidonic acid are shown in Fig. 1. The cyclooxygenase

![Graph showing radioactivity from synthesized compounds]

Fig. 1: Synthesis of HETE, HHT and TXB$_2$ by rabbit platelets (200 µg protein) incubated with arachidonic acid (20:4) in the absence of indomethacin. The incubation system is described under MATERIALS AND METHODS. The LK6D plate was developed with ethyl acetate/2,2,4-trimethylpentane/water (75:75:100, upper phase) and scanned for radioactivity.
pathway is inhibited by indomethacin and only one product is identified when platelets are incubated with arachidonic acid in the presence of indomethacin (Fig. 2). It should be noted that a second

![Diagram]

**Fig. 2:** Synthesis of HETE by rabbit platelets incubated with arachidonic acid (20:4) in the presence of indomethacin. The incubation system is described under MATERIALS AND METHODS. The LK6D plate was developed with ethyl acetate/2,2,4-trimethylpentane/acetic acid (100:100:1) and scanned for radioactivity. A: 50 μg platelet protein; B: 250 μg platelet protein.

lipoxygenase product, trihydroxyeicosatetraenoic acid (THETE), is formed only at a much higher arachidonic acid concentration (Bryant and Bailey, 1979) than the concentration used in our study.
Several observations show that the incubation product is HETE synthesized by lipoxygenase rather than a nonenzymatic autooxidation product of arachidonic acid. Thus 15-hydroxyeicosatetraenoic acid, synthesized with soybean lipoxygenase, has the same $R_f$ value as the incubation product. The incubation product separates as a single spot with several TLC systems including a silver nitrate plate developed with ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90:50: 20:100, upper phase). TYA, an inhibitor that inhibits HETE production in platelets (Hamberg and Samuelsson, 1974), abolished the formation of the incubation product. The incubation produce was not obtained with boiled platelets. Product formation was time dependent, increasing from 4% of 0 time to 75% after a 15 min incubation period. Finally, a potassium iodide-starch spray showed that the product did not contain a visible hydroperoxide.

**Lipoxygenase Activity in Platelets from Rabbits on Standard, Vitamin E-Deficient and Vitamin E-Supplemented Dietary Regimens:** Platelets were isolated and the washed platelets, at several concentrations, were incubated with arachidonic acid. Unreacted arachidonic acid and HETE were measured. At 10 μg of platelet protein, vitamin E-supplemented rabbits had significantly greater amounts of unreacted arachidonic acid and significantly smaller amounts of HETE than platelets from standard or vitamin E-deficient rabbits (Table 1). These differences tended to disappear at higher platelets concentrations. At 20 μg of platelet protein, the only significant difference was found in the recovery of unreacted arachidonic acid from vitamin E-deficient and vitamin E-supplemented rabbits (Table 1). Even this difference disappeared at a
higher platelet concentration (Table 1). These data show that a vitamin E-supplemented diet has a significant inhibitory effect on platelet lipoxygenase activity and that this effect is only apparent at a low platelet concentration or region of substrate excess.

**Table 1. Effect of Vitamin E Supplemented and Deficient Diets on HETE Synthesis from Arachidonic Acid in Washed Platelets.**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Platelet protein (µg)</th>
<th>Arachidonic acid (pmoles/15 min)</th>
<th>HETE Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard (5)b</td>
<td>10.2 ± 0.7</td>
<td>285 ± 22c</td>
<td>149 ± 22c</td>
</tr>
<tr>
<td>Deficient (3)</td>
<td>11.0 ± 1.3</td>
<td>248 ± 15c</td>
<td>169 ± 10c</td>
</tr>
<tr>
<td>Supplement (6)</td>
<td>9.0 ± 1.1</td>
<td>363 ± 24c</td>
<td>79 ± 17c</td>
</tr>
<tr>
<td>Standard (5)</td>
<td>20.4 ± 1.4</td>
<td>252 ± 19</td>
<td>184 ± 21</td>
</tr>
<tr>
<td>Deficient (4)</td>
<td>20.6 ± 3.7</td>
<td>212 ± 9d</td>
<td>203 ± 9</td>
</tr>
<tr>
<td>Supplemented (4)</td>
<td>22.1 ± 1.9</td>
<td>277 ± 29d</td>
<td>156 ± 36</td>
</tr>
<tr>
<td>Deficient (3)</td>
<td>226.0 ± 17.0</td>
<td>75 ± 62</td>
<td>347 ± 48</td>
</tr>
<tr>
<td>Supplemented (5)</td>
<td>239.0 ± 6.0</td>
<td>121 ± 62</td>
<td>310 ± 60</td>
</tr>
</tbody>
</table>

a The incubation mixtures contained washed platelets (protein content specified) and arachidonic acid (460 pmoles) in 0.5 ml of buffer containing 0.3 mM indomethacin.

b Number of animals are in parentheses.

c The Student "t" test showed that standard, deficient and supplemented diets differed significantly, P<0.005.

d The Student "t" test showed that deficient and supplemented diets differed significantly, P<0.005.

**Lipoxygenase Activity in Platelets Preincubated with Vitamin E:**

Washed platelets were preincubated for 5 min with vitamin E and then assayed for lipoxygenase activity. Vitamin E inhibited lipoxygenase in platelets from normal human subjects and platelets from vitamin E-deficient rabbits. The recoveries of unreacted arachidonic acid and HETE were proportional to the vitamin E added, in vitro, to the
incubation system (Table 2). Species and nutritional status did not alter the inhibitory effect of vitamin E. Preincubation was necessary for inhibition with vitamin E.

Table 2. Effects of Vitamin E, Vitamin E Acetate, and Tween 20 on HETE Synthesis from Arachidonic Acid in Washed Platelets.\(^a\)

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Arachidonic acid (pmoles/15 min)</th>
<th>HETE</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (normal human)</td>
<td>210 ± 36(^b)</td>
<td>192 ± 30</td>
<td></td>
</tr>
<tr>
<td>+ Vitamin E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X 10(^{-5})M</td>
<td>257(^c)</td>
<td>169</td>
<td>12</td>
</tr>
<tr>
<td>2 X 10(^{-3})M</td>
<td>407</td>
<td>45</td>
<td>77</td>
</tr>
<tr>
<td>+ Vitamin E acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X 10(^{-5})M</td>
<td>263</td>
<td>167</td>
<td>13</td>
</tr>
<tr>
<td>2 X 10(^{-3})M</td>
<td>414</td>
<td>40</td>
<td>79</td>
</tr>
<tr>
<td>+ 1% Tween 20</td>
<td>419 ± 31</td>
<td>10 ± 6</td>
<td>95</td>
</tr>
<tr>
<td>Control (Vitamin E-deficient rabbit)</td>
<td>212 ± 9</td>
<td>203 ± 9</td>
<td></td>
</tr>
<tr>
<td>+ Vitamin E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X 10(^{-5})M</td>
<td>293</td>
<td>123</td>
<td>49</td>
</tr>
<tr>
<td>2 X 10(^{-4})M</td>
<td>410</td>
<td>38</td>
<td>81</td>
</tr>
<tr>
<td>2 X 10(^{-3})M</td>
<td>432</td>
<td>23</td>
<td>89</td>
</tr>
<tr>
<td>+ Vitamin E acetate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 X 10(^{-5})M</td>
<td>288</td>
<td>121</td>
<td>40</td>
</tr>
<tr>
<td>2 X 10(^{-4})M</td>
<td>374</td>
<td>62</td>
<td>69</td>
</tr>
<tr>
<td>2 X 10(^{-3})M</td>
<td>428</td>
<td>21</td>
<td>90</td>
</tr>
</tbody>
</table>

\(^a\) The incubation mixtures contained washed platelet (20 µg protein) and arachidonic acid (460 pmoles) in 0.5 ml of buffer containing 0.3 mM indomethacin.

\(^b\) Mean ± S.D.

\(^c\) Average of 2 determinations.

Lipoxygenase Activity in Platelets Preincubated with Vitamin E Acetate: A derivative of vitamin E, vitamin E acetate, inhibited HETE
production in platelets as effectively as vitamin E (Table 2). Since platelets may contain enzymes that hydrolyze vitamin E acetate to vitamin E, incubation mixtures containing vitamin E acetate were extracted and analyzed by HPLC for the formation of vitamin E (Fig. 3).

Fig. 3: HPLC analysis of the hexane extract from an incubation mixture containing $2 \times 10^{-3}$ M vitamin E acetate, 100 µg platelet protein, 0.3 mM indomethacin, and 460 pmoles of arachidonic acid. The incubation system and HPLC separation are described under MATERIALS AND METHODS.

No vitamin E (elution time 9.7 min) was detected. Only a trace amount of vitamin E quinone corresponding to the quinone in unreacted vitamin E acetate was found. The data show that vitamin E acetate is the actual inhibitor in these experiments.
Lipoxygenase Activity in Platelets Preincubated with Tween 20: A washed platelet suspension from human platelets was mixed with 1% Tween 20 and assayed for lipoxygenase activity. The detergent inhibited the enzyme and over 90% of the substrate was recovered as unreacted arachidonic acid (Table 2).

DISCUSSION

Studies involving the dietary manipulation of vitamin E and studies involving preincubation, in vitro, with vitamin E both show that platelet lipoxygenase is inhibited by this antioxidant. Since lipoxygenase is highly active in platelets, the inhibitory effect of vitamin E is apparent only at low platelet concentrations. In a previous study Rao et al. (1978) found that a mixture of nitroblue tetrazolium and vitamin E inhibited HETE production from platelets. A later study from the same laboratory (Butler et al., 1979) showed that vitamin E alone had no effect on HETE production. The data in that study are in direct conflict with the data generated in the present study. We have found that vitamin E has little effect on HETE production when the platelet protein concentration is increased 10-fold. The platelet concentration used by Butler et al. (1979) may have been too high for vitamin E to exert a significant inhibitory effect since these investigators apparently worked at a much higher platelet concentration in their studies.

Vitamin E is a surfactant and surfactants concentrate at hydrophilic-hydrophobic interfaces such as cell membranes (Fukuzawa et al. 1977); Maggio et al., 1977; Patil and Cornwell, 1978). Acetate derivatives also behave as surfactants (Kwong et al., 1971). Vitamin E
may function as an antioxidant either through its scavenger effect on free radicals or its surfactant effect on the cell membrane (Menzel, 1976; Lucy, 1978). These effects have been distinguished in recent studies which compare the antioxidant properties of vitamin E (radical scavenger and surfactant) and vitamin E acetate (surfactant) in the non-enzymatic peroxidation of liposomes (Gutteridge, 1978) and red cells (Mino and Sugita, 1978). Since vitamin E and vitamin E acetate both inhibited lipid peroxidation, it appeared that these compounds functioned through the effect that a surfactant had on the lipid bilayer (liposome) or cell membrane (red cell).

Surfactant studies were extended to enzymatic peroxidation in the present investigation. Vitamin E and vitamin E acetate, in vitro, were equally effective inhibitors of platelet lipoxygenase. One percent Tween 20 was a highly effective inhibitor of platelet lipoxygenase (Table 2) while 0.5% Tween 20 had no effect (Dutilh et al., 1979). These data show that vitamin E functions, in vitro, as a surfactant in the inhibition of platelet lipoxygenase. These data suggest that vitamin E may function, in vivo, as a surfactant in the inhibition of platelet lipoxygenase. It is interesting that vitamin E has little effect on microsomal prostaglandin synthetase (Panganamala et al., 1977) and that prostaglandin synthetase is not inhibited by 1% Tween 20 (Marnett et al., 1977; Panganamala et al., 1979).

ACKNOWLEDGEMENTS

This study was supported in part by Research Grants HL-11897 and HL-22439 from the National Institutes of Health, and a grant from the National Livestock and Meat Board. We appreciate the technical assistance of Mrs. G. Devaraju and Miss N. J. Dorman.
REFERENCES


RELATIVE ABILITIES ON A MOLAR BASIS OF HEXAFLUORO-, HEXACHLORO-
AND HEXABROMOBENZENES TO DECREASE LIVER UROPORPHYRINOGEN
DECARBOXYLASE ACTIVITY AND CAUSE PORPHYRIA IN FEMALE RATS

Andrew G. Smith and Jean E. Francis

Biochemical Pharmacology Section,
MRC Toxicology Unit,
Medical Research Council Laboratories,
Woodmansterne Road,
Carshalton (SM5 4BF)
Surrey, U.K.

ABSTRACT

Virgin female Agus rats were fed a diet containing hexahalo-
genated benzenes (700 nmol/g of food) for 70 days. The liver
concentrations of these compounds were then determined and corre-
lated with any depression in uroporphyrinogen decarboxylase activity
and increases in porphyrin levels (indicating the induction of
porphyria). Hepatic concentrations of the compounds corresponded
to the series F > Cl > Br. However, depression of decarboxylase
activity and increase in porphyrin levels were Cl > Br > F,
(hexachlorobenzene and hexabromobenzene increased porphyrins by
513- and 17-fold respectively) suggesting that at its site of action
in the liver hexabromobenzene may be more porphyrrogenic than the
chlorine analogue. This may suggest a relationship between the
strength of the carbon-halogen bond and the induction of porphyria.

INTRODUCTION

Hexachlorobenzene (and some other polychlorinated aromatic
compounds) causes a chronic hepatic porphyria characterised by an
accumulation of uroporphyrin and 7-carboxylic porphyrin. This is
probably the consequence of a decrease in the activity of liver
uroporphyrinogen decarboxylase (Elder, 1978). On the other hand,
Carlson (1979) has reported that hexabromobenzene, a fire retardant,
is an extremely poor inducer of porphyria relative to hexachloro-
benzene (Carlson, 1978). Only a 45% increase in liver porphyrins
was observed after oral dosing of female rats with 200 mg/kg daily
for 120 days and Mendoza et al. (1977) could find no significant
increase in liver porphyrins at all in male rats fed this compound. Direct comparisons with hexachlorobenzene were not made in either experiment. However, Mendoza et al. (1979) have directly compared these two chemicals using primiparous rats and found that the bromine compound appeared to be much more effective in inducing porphyria than the chlorine analogue. Although Mendoza et al. measured the liver concentrations of the two halogenated benzenes these were compared on a weight for weight basis and the significantly different molecular weights were not taken into account.

In view of this omission and the apparent discrepancy from the results of Carlson (1979), we have investigated the relative abilities on a molar basis, of hexafluoro-, hexachloro- and hexabromo-benzenes to cause porphyria in virgin female rats. The hepatic concentrations of the benzenes have been correlated with the levels of uroporphyrinogen decarboxylase and the degrees of porphyria in order to assess more accurately their relative toxicities.

MATERIALS AND METHODS

Chemicals

Hexachlorobenzene (Organic Analytical Grade) was obtained from B.D.H. Chemicals Co.Ltd., Poole, Dorset, U.K. Hexafluorobenzene (99%), hexabromobenzene (98%), pentachlorobenzene (98%) and pentachloropyridine (98%) were purchased from Aldrich Chemical Co.Ltd., Gillingham, Dorset, U.K. Pentachloronitrobenzene virtually free from hexachlorobenzene was a gift from Dr. J.R.P. Cabral.

Animals and treatment

Virgin female rats (35-45 days old) were of the inbred Agus strain (Festing and Staats, 1973). They were fed for 70 days on a powdered diet 41B (Labsure Animal Foods, Poole, Dorset, U.K.) normally containing 2% arachis oil and supplemented with 700 nmol of halogenated benzene/g of food (equivalent to 200 ppm of hexachlorobenzene). Diet containing hexafluorobenzene was only given in small amounts at a time to minimize any possible losses by evaporation.
Analyses

The rats were killed by decapitation and the livers removed, rinsed, blotted and weighed. The median lobes were then homogenised in 0.1M NaHPO$_4$ – NaH$_2$PO$_4$ buffer (pH 6.8) containing 0.1 mM disodium EDTA (1:4 w/v), and portions of the homogenates were analysed as described below.

Porphyrians These were estimated as uroporphyrin as described previously (Smith et al., 1979) using a spectrofluorometric method (Granick et al., 1975).

Uroporphyrinogen decarboxylase This was assayed by the method of Smith and Francis (1979) using uroporphyrin (approx. 80% isomer III isolated from the livers of HCB-fed rats). The porphyrin was reduced and the uroporphyrinogen formed (8 nmol) was incubated for 1 h with 0.2 ml of homogenate and 0.75 ml of the above buffer system containing 3 µmol of thioglycollate. The coproporphyrinogen produced was then estimated.

Halogenated benzenes An acetonitrile-hexane extraction procedure (for 3 h) was employed for hexachloro-, hexabromo- and pentachloronitrobenzenes (Mendoza et al., 1979) and the hexane layers analysed by electron capture gas chromatography (Smith et al., 1979) using a 1.5 m 3% Dexsil 300 column at 200 or 230°C. Hexabromobenzene proved more difficult to assay but this was finally accomplished by means of extraction with toluene and chromatography on a 2m Porapak Q column at 175°C.

RESULTS

Young female rats were fed a diet containing 700 nmol of halogenated benzene/g of food. This is equivalent to 200 ppm of hexachlorobenzene and at the end of the experiment the animals were consuming approximately 10-15 µmol daily. After 70 days they were killed and the livers removed. The median lobes, which show the fastest development of porphyria (A.G. Smith, J.E. Francis and F. De Matteis, unpublished data) were homogenised and the levels of the fed compound, porphyrin and uroporphyrinogen decarboxylase were determined.

Table 1 shows that hexachlorobenzene was the more effective inducer of an increase in liver size although hexabromobenzene also had a noticeable effect. Hexafluorobenzene and
TABLE 1
EFFECTS OF POLYHALOGENATED BENZENES ON THE BODY AND LIVER WEIGHTS OF FEMALE AGUS RATS

<table>
<thead>
<tr>
<th>Treatment (No.of animals)</th>
<th>Mol.wt of compound</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>Liver levels of compound (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7)</td>
<td>-</td>
<td>195 ± 4</td>
<td>6.9 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Hexafluorobenzene (7)</td>
<td>186</td>
<td>195 ± 4</td>
<td>6.7 ± 0.3</td>
<td>3141 ± 350</td>
</tr>
<tr>
<td>Hexachlorobenzene (7)</td>
<td>284</td>
<td>207 ± 6</td>
<td>8.6 ± 0.2***</td>
<td>442 ± 45</td>
</tr>
<tr>
<td>same (4)†</td>
<td></td>
<td>298 ± 3</td>
<td>8.3 ± 0.6*</td>
<td>476 ± 40</td>
</tr>
<tr>
<td>Hexabromobenzene (7)</td>
<td>552</td>
<td>196 ± 3</td>
<td>7.6 ± 0.2**</td>
<td>32.2 ± 2.7</td>
</tr>
<tr>
<td>same (5)†</td>
<td></td>
<td>200 ± 4</td>
<td>8.2 ± 0.2***</td>
<td>30.9 ± 2.4</td>
</tr>
<tr>
<td>Pentachloronitrobenezene (5)</td>
<td>295</td>
<td>193 ± 4</td>
<td>6.8 ± 0.3</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

Rats (35-45 days old) were given food containing 700 nmol of compound/g for 70 days.
† Compound given in diet containing 5% oil. All other diets contained 2% oil.
Values are means ± S.E.M. *P < 0.05, **P < 0.025, ***P < 0.001.
The molecular weights quoted are those of the major parent ions in the electron impact mass spectra.
pentachloronitrobenzene were without effect. The liver levels of the hexahalogenated benzenes were related to the halogen series (a 100-fold difference between fluorine and bromine-analogues). This probably reflects the high lipid solubility of hexafluorobenzene (a liquid at room temperature) compared to the low solubility of hexabromobenzene in aqueous and organic solvents. The even lower concentration of pentachloronitrobenzene may reflect a poor absorption but this compound is also likely to be excreted rapidly after conjugation through the nitro function.

To investigate the porphyrogenicity of these compounds the uroporphyrinogen decarboxylase and porphyrin levels were measured (Table 2). Hexafluorobenzene had no effect. Hexabromobenzene

TABLE 2

LIVER UROPORPHYRINOGEN DECARBOXYLASE ACTIVITIES AND PORPHYRIN LEVELS

IN FEMALE AGUS RATS FED HALOGENATED BENZENES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uroporphyrinogen decarboxylase (nmol/h/g)</th>
<th>Porphyrins (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.7 ± 0.6</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Hexafluorobenzene</td>
<td>26.6 ± 1.0</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>7.0 ± 0.6***</td>
<td>190.6 ± 49.4**</td>
</tr>
<tr>
<td>same †</td>
<td>12.6 ± 1.6***</td>
<td>32.5 ± 5.6***</td>
</tr>
<tr>
<td>Hexabromobenzene</td>
<td>20.9 ± 1.9</td>
<td>6.5 ± 2.4*</td>
</tr>
<tr>
<td>same †</td>
<td>25.5 ± 0.3</td>
<td>3.3 ± 0.8**</td>
</tr>
<tr>
<td>Pentachloronitrobenzene</td>
<td>22.8 ± 0.5</td>
<td>0.4 ± 0.01*</td>
</tr>
</tbody>
</table>

Animals treated as described in Table 1. † 5% oil in diet. Values are means ± S.E.M. *P < 0.025, **P < 0.005, ***P < 0.001. Uroporphyrinogen decarboxylase activity is expressed as nmol of coproporphyrinogen formed. Porphyrins are estimated as uroporphyrin.

caused a small decrease in decarboxylase activity with a resulting 17-fold increase in porphyrins. Hexachlorobenzene was however, by
far the most potent agent, with uroporphyrinogen decarboxylase decreased by 70% and porphyrin levels increased 513-fold. Raising the oil content of the diet from 2% to 5% made no difference to the liver levels of hexachlorobenzene and hexabromobenzene (Table 1) but the degrees of porphyria were apparently lower in both cases (Table 2). However, the animals fed the former compound showed considerably more irritation of the skin than usual.

Pentachloropyridine which might be thought to possess similar porphyria-inducing properties to hexachlorobenzene, being a fully halogenated aromatic compound, caused only a small increase in liver porphyrins similar to that observed with pentachlorobenzene (0.69 ± 0.02 and 0.67 ± 0.02 nmol/g, respectively, for 5 animals in each experiment).

**DISCUSSION**

In our experiments hexachlorobenzene fed in the diet was clearly much more effective than hexabromobenzene in causing an increase in liver size and producing porphyria, as suggested by the results of Carlson (1979). However, hexabromobenzene did elicit a significant response but not greater than hexachlorobenzene as observed by Mendoza et al. (1979). If the liver concentrations of the halogenated benzenes are taken into account when considering the porphyrin levels, and thereby excluding different rates of intestinal absorption, it may be concluded that at its site of action the specific activity of hexabromobenzene is in fact greater than that of its chlorine analogue, with hexafluorobenzene appearing to have no porphyrogenic effect. If this is true there are two possible implications as far as the mechanism of action is concerned. The
size and polarity of these compounds may be critical in inducing some kind of biochemical change, although it is difficult to see how the porphyria-inducing chemicals 2,3,7,8-tetrachlorodibenzop-dioxin (Goldstein et al. 1973) and chlorinated biphenyls (Goldstein et al. 1974) fit into this scheme. Alternatively, the ability of a compound to cause chronic hepatic porphyria may be related in some way to the ease of cleavage of a carbon-halogen bond i.e. C-Br > C-Cl > C-F. The low porphyrogenicity of the pentachloro chemicals studied may arise by the predominance of other routes of metabolism.

We are unable to explain why in the studies of Mendoza et al. (1979) hexabromobenzene was such a good inducer of porphyria relative to hexachlorobenzene and yet was present in the livers at much lower concentrations (1:8 w/w). Perhaps in primiparous rats some biochemical changes occur which increase metabolism of the brominated benzene. In our investigations, increasing the dietary oil content did not change the uptake of these compounds or alter their relative toxicities.

ACKNOWLEDGMENTS

We would like to thank Dr.F. De Matteis for his interest and advice and Dr. E. Bailey for helpful discussions.

REFERENCES


