Induction of microsomal drug metabolism in man and in the rat by exposure to petroleum

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ABSTRACT To determine the effect of petroleum exposure on the activity of hepatic mixed function oxidase enzymes, salivary elimination kinetics of antipyrine were determined in 19 petrol station attendants and compared with 19 controls. Antipyrine half life in petrol station attendants was shorter than in controls. Microsomal preparations ($10\,000 \times g$ supernatants) were prepared from six male Porton rats exposed to petrol vapour (5 ppm at an air flow rate of 4 l/min for eight hours a day for three weeks) and six control rats maintained under the same conditions without exposure to petrol vapour. The rates of oxidative metabolism of antipyrine, aminopyrine, ethylmorphine, aniline, and benzo(a)pyrene were all increased by more than 45% in the petrol-exposed rats. The results indicate that petrol vapour is a moderately potent inducer of mixed function oxidase activity in rats, and that occupational exposure to petroleum may result in enhanced microsomal drug metabolism.

The activity of the hepatic mixed function oxygenases may be induced by many heterogeneous lipophilic compounds. Such chemicals are proliferating in the environment, and it is of some interest to know which of these agents are capable of causing microsomal enzyme induction in man.

Several environmental chemicals are known to be able to cause microsomal enzyme induction in animals.^{1,2} Among these are pesticides, such as DDT, chlordane and dieldrin, and polycyclic hydrocarbons, such as 3-methylcholanthrene and 3,4 benzo(a)pyrene. Evidence for enzyme-inducing effects of environmental chemicals in man is more limited. Changes in antipyrine elimination kinetics consistent with induced metabolism, however, have been detected in man after occupational exposure to chlorinated hydrocarbon insecticides,³ polychlorinated biphenyls,⁴ and inhalational anaesthetics.⁵

Petroleum is a complex mixture of a wide variety of lipophilic hydrocarbons, some of which may have the potential to induce mixed function oxidase activity. If this were the case enzyme induction might be detectable in workers occupationally ex-

posed to petroleum. A convenient method of assessing such induction entails the use of antipyrine as a metabolic probe combined with salivary sampling as a non-invasive procedure for sample collection.⁶

In the present study the kinetics of antipyrine elimination were determined in petrol station attendants, and an attempt was made to correlate this with the degree of exposure to petrol, using urinary phenol output and blood and urinary lead concentrations as indices of exposure. In addition the inductive effect of petrol on microsomal drug metabolising enzyme activity *in vitro* was assessed in rats after three weeks' intermittent exposure to petrol vapour.

Methods

HUMAN STUDY

Subjects

Nineteen male employees at six metropolitan petrol-vending stations participated in the study. All had worked as petrol pump attendants or garage mechanics, or both, for more than one year. They were aged 16-50 (mean $\pm$ SD, 28 ± 10), and they were not taking any medicine at the time of the study. On the morning of the third day of a five-and-a-half-day working week, each subject ingested

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antipyrine (10 mg/kg) dissolved in 100 ml of water. Mixed saliva samples were collected before and 3, 6, 9, 12, 24, and 36 hours after antipyrine administration. Antipyrine concentration in saliva was assayed by a gas-liquid chromatographic technique as previously described.⁷ Ambient temperature at the time of the experiment was 13–19°C. The control group consisted of 19 unmedicated men, mostly university staff and students, aged 21–69 (mean $\pm$ SD, 29 $\pm$ 12), whose exposure to petroleum products was limited to personal use of a motor vehicle.

Thirteen of the petrol station attendants and ten of the controls also participated in a study designed to assess exposure to petroleum products. On the morning before the antipyrine study, a 20-ml venous blood sample was collected into lithium heparin (125 IU/10 ml). On the following morning, they began a 24-hr urine collection into plastic bottles containing 50 ml of 2M nitric acid as a preservative. The blood and urine samples were analysed as follows.

Urine

Phenol—Total urinary phenol output (mg/24 hr) was determined by the distillation method of Schmidt.⁸

Lead—The 24-hr urinary lead output was measured by direct solvent extraction and atomic absorption spectroscopy (Standards Association of Australia, 1970).

Blood

Aminolaevulinic acid dehydratase (ALAD) activity—ALAD concentrations were assayed in whole blood by the colorimetric method described by Chakrabarti *et al.*,⁹ except that incubation was performed in evacuated Thunberg tubes for 30 min.

Blood lead—One millilitre of whole EDTA blood was mixed with 4 ml of 0.25% Triton X-100 and atomic absorption analysis performed as described by Fernandez.¹⁰

Blood biochemistry—Biochemical analysis was performed using standard Technicon autoanalyser techniques. Items measured included sodium, potassium, chloride, bicarbonate, osmolality, glucose, urea, creatinine, urate, phosphate, calcium, albumin, globulins, cholesterol, conjugated and total bilirubin, as well as alkaline phosphatase, lactic dehydrogenase, aspartate transaminase, and creatinine kinase activities.

ANIMAL STUDY

Animals

Male Porton rats, weighing 100–150 g, were obtained from this university's animal breeding house. They

were kept in a 12-hr light/dark cycle and allowed food and water *ad libitum*.

Petrol exposure

Perspex exposure chambers of about 40-l capacity were supplied with an airflow of 4 l/min from a respiratory pump (C F Palmer, London). The airflow from the pump was split, with half flowing to the chamber housing six control rats and the other passing to a similar chamber via a petrol vaporising chamber that consisted of a water heated, vertical 22 $\times$ 4 cm Pyrex tube. The middle 16 cm had a glass spiral tube through which water at 40°C was pumped. Petrol (commercial grade, 50% super: 50% standard) was pumped on to the top of the spiral at a rate of 20 mg/min and allowed to evaporate. Thus the six rats in this chamber were exposed to petrol vapour at a calculated concentration of 5 mg/l. Both groups of rats were placed in the exposure chambers for eight hours a day for three weeks.

In-vitro microsomal enzyme activities

Rats were killed by stunning and decapitation. Blood samples (2 ml) were collected into heparinised tubes. The livers were perfused *in situ* with ice-cold saline via the portal vein and placed in 20 ml of ice-cold 0.25M sucrose/0.05M Tris buffer (pH 7.4). All subsequent operations were carried out at 0–4°C. The livers were weighed and homogenised for 1 min using an Ultra Turrax blender. The homogenate was centrifuged for 20 min at 10 000 $\times$ g, and the resulting supernatant decanted and the volume adjusted to 400 mg liver/ml with the sucrose/Tris buffer. The incubation mixture (total volume 3 ml) consisted of 0.5 ml of the 10 000 $\times$ g supernatant (pre-incubated for 2 min) and 5 mmol/l MgCl₂, 0.33 mmol/l NADP⁺, 3.3 mmol/l glucose-6-phosphate, and substrate in 0.05M Tris buffer pH 7.4. The incubations were carried out in glass vials in a metabolic shaker bath at 37°C under air at 80 oscillations/min with a glass marble to facilitate mixing. Substrate concentrations were 5 mmol/l for aminopyrine, ethylmorphine, aniline, and antipyrine and 80 μ mol/l for benzo(a)pyrene (added as 15 μ l of a 4 mg/ml solution in acetone).

Aminopyrine and ethylmorphine N-demethylase activities and aniline p-hydroxylase activity were determined as described by Schenkman *et al.*,¹¹ except that semicarbazide (5 mmol/l) was added to trap formaldehyde formed during demethylation. Benzo(a)pyrene hydroxylase activity was determined using the method of Nebert and Gelboin¹² with the following modification. The incubation was stopped by the addition of 3 ml ice-cold acetone, and 3 ml of this mixture was extracted with 10 ml n-hexane in a 20-ml glass extraction tube for 10 min and then

centrifuged for 5 min at 3000 rpm. Three millilitres of the organic phase was back extracted into 3 ml 1M NaOH, and the fluorescence of the aqueous phase read at 522 nm with the excitation at 396 nm in a spectrofluorimeter (Farrand Optical, New York). In the blank, substrate was added after incubation. Antipyrine metabolism was determined as the amount of water soluble metabolites produced during incubation. The antipyrine used as substrate was spiked with N-methyl- ^{14}C -antipyrine (2.2×10^5 dpm/vial). The reaction was stopped with 0.6 ml 5M NaOH and 3 ml of this mixture was extracted with 10 ml chloroform for 30 min. Only unchanged antipyrine was extracted into the organic phase,¹³ leaving the metabolites in the aqueous phase. After centrifugation for 30 min at 3000 rpm, 2 ml of the aqueous medium was transferred to a plastic counting vial containing 10 ml of scintillant (0.17 g 1,4-bis (2(4-methyl-5-phenyloxazolyl)) benzene, 5.3 g 2,5-diphenyloxazole, 333 ml Triton X-100, 666 ml toluene) and the activity determined in a liquid scintillation counter (Packard, Model 3310). In the blank, substrate was added after incubation. The amount of antipyrine metabolised, allowing for volume corrections, was determined as follows: amount metabolised =

$$\frac{\text{dpm in aqueous phase} - \text{dpm in blank}}{\text{total dpm added}} \times \text{initial amount in the incubation vial.}$$

Incubation times were 10 min for ethylmorphine and benzo(a)pyrene, 15 min for aminopyrine and aniline, and 30 min for antipyrine. Aniline aminotransferase (ALAT) activity and bilirubin content of rat plasma were assayed by standard Technicon autoanalyser techniques.

ANALYSIS OF DATA

Comparisons of grouped data were made using the Mann Whitney U-test. The Spearman rank correlation was used to test correlation, and the classification measurements were made using chi-squared analysis. Non-parametric confidence limits were determined as described by Colquhoun.¹⁴

Results

HUMAN STUDY

Antipyrine metabolism

Antipyrine, 10 mg/kg, was given as a single oral dose to each subject and the elimination rate of antipyrine determined. As illustrated in fig 1, the half lives in petrol station attendants ranged from 4.2 to 17.9 hr (median 9.7 hr), and these values were shorter than in the male control subjects whose values ranged

Fig 1 Antipyrine half-lives in petrol station workers and control subjects. Median value of each group is shown by a bar.

from 7.1 to 16.5 hr (median 11.3 hr) ($p < 0.05$, one-tail).

Urinary phenol

The 24-hr urinary excretion of phenol in the 13 petrol station attendants who participated in the blood and urine collections ranged from 4.8 to 34.1 mg (median 12.2 mg, with 98% confidence limits of 6.6 and 21.0 mg). These values (fig 2) were not different from those found in 10 control subjects, whose values ranged from 5.7 to 15.6 mg (median 8.8 mg, with 98% confidence limits of 5.9 and 14.0 mg) ($p > 0.05$, one-tail). The values obtained in the control subjects are similar to those found in urine from normal subjects by Schmidt.⁸ There was no rank correlation between antipyrine half life and phenol excretion ($r_s = 0.29$, $p > 0.05$).

Lead content

Values for blood and urinary lead and blood ALAD activity in the 13 workers are shown in table 1. The urinary lead output of the petrol station attendants ranged from 0.04 to 0.20 $\mu\text{mol}/24$ hr (median 0.07,

Fig 2 Amount of phenol excreted in 24-hour urine samples from 13 petrol station workers and ten controls. Median value of each group is shown by a bar.

with 98% confidence limits of 0.06 and 0.17) and the blood lead concentrations ranged from 0.79 to 1.56 $\mu\text{mol/l}$ (median 0.95, with 98% confidence

Table 1 Values for blood and urinary lead concentrations and plasma ALAD activity in 13 petrol station attendants

Subject	Blood*	Lead level	
		Urine†	ALAD‡
1	1.04	0.08	7.5
2	0.80	0.06	14.5
3	0.79	0.04	15.8
4	0.91	0.15	11.9
5	1.15	0.07	7.1
6	0.82	0.07	11.9
7	0.95	0.06	16.9
8	0.79	0.12	16.6
9	1.56	0.20	11.8
10	1.42	0.12	4.3
11	1.03	0.05	15.9
12	1.15	0.07	10.4
13	1.03	0.17	13.6
Mean	1.03	0.10	11.6
SD	0.24	0.05	3.8
Normal range of controls	< 1.93	(0.10-0.50)	(7.9-19.1)

* $\mu\text{mol/l}$.

† $\mu\text{mol/24 hr}$.

‡nmol porphobilinogen formed/min/ml RBC.

limits of 0.80 and 1.15). These values are all well within the range (urine 0.10-0.50 $\mu\text{mol/24 hr}$ and blood 1.7 $\mu\text{mol/l}$ limit) quoted for normal non-exposed subjects.¹⁵ The levels of ALAD activity in the petrol station workers were consistent with the finding of normal values for blood and urinary lead ranging from 4.25 to 16.9 nmol porphobilinogen formed/min/ml RBC, with a median of 11.8 and 98% confidence limits of 7.5 and 15.8. These values are comparable to the mean value of 13.5 nmol porphobilinogen formed/min/ml RBC in 37 subjects with no known exposure to lead.¹⁶ There were no significant rank correlations between antipyrine half life and either urinary lead ($r_s = 0.14$, $p > 0.05$), blood lead ($r_s = 0.40$, $p > 0.05$), or ALAD ($r_s = 0.19$, $p > 0.05$). A significant correlation, however, was found between blood lead concentrations and ALAD activity ($r_s = 0.48$, $p < 0.05$), a result consistent with previous findings.^{9 16 17}

These results indicate that the petrol station workers did not accumulate lead to a greater extent than that expected in a normal population group.

Blood biochemistry

No abnormalities were disclosed by the biochemical tests of blood chemistry in the 13 petrol station attendants.

Social history

Consideration was given to the possibility that factors such as age, duration of employment, smoking habits, alcohol, and caffeine intake may have had some influence on antipyrine metabolism in the 19 petrol station attendants (table 2). No association was found between antipyrine half life and any of these factors.

ANIMAL STUDY

As shown in table 3, the activity of mixed function oxidases measured in the microsome-enriched 10 000 $\times$ g supernatant preparations was increased in the petrol-exposed rats. The percentage increase ranged from 45% for benzo(a)pyrene hydroxylation to 79% for ethylmorphine N-demethylation. All these increases were highly significant ($p < 0.001$, two-tail). The liver/body weight ratio was also increased in the petrol-exposed rats ($p < 0.01$, two-tail), although body weights in the two groups were not different ($p > 0.1$, two-tail), nor was the increase in body weight over the 21-day exposure (89% in control v 75% in petrol exposure).

There was no difference in plasma alkaline phosphatase and bilirubin concentrations between control and exposed rats ($p > 0.1$, two-tail), suggesting that exposure to petrol did not cause overt liver damage. The values for bilirubin were $0.45 \pm$

Table 2 Antipyrine half-lives (hours), age and work duration (years) and measures of social drug intake in 19 petrol station attendants

Subject	Half-life	Age	Work duration	Alcohol* intake	Caffeine† intake	Smoking‡
1	4.2	20	4	0	1	0
2	6.8	16	1	1	2	1
3	7.8	28	11	1	1	1
4	8.4	33	18	2	2	0
5	8.4	47	33	0	2	1
6	8.4	22	7	1	1	0
7	8.7	19	3	2	1	1
8	9.1	23	7	1	1	0
9	9.1	42	20	1	2	1
10	9.7	19	3	1	1	0
11	10.2	17	2	0	0	0
12	10.2	42	25	0	2	0
13	10.4	27	10	1	1	0
14	10.5	50	10	2	2	1
15	10.8	30	12	2	1	2
16	10.9	20	2	0	1	0
17	11.3	21	4	1	1	1
18	12.1	27	10	0	1	0
19	17.9	30	15	1	2	0
Correlation coefficient		0.1 p > 0.1	0.06 p > 0.1			
Chi-square				0.5 p > 0.5	0.36 p > 0.5	0.44 p > 0.5

*0 = no alcohol; 1 = ≤ 1 bottle beer/day or equivalent; 2 = > 1 bottle beer/day.†0 = no coffee or tea; 1 = ≤ 5 cups coffee or tea/day; 2 = > 5 cups coffee or tea/day.‡0 = non-smoker; 1 = ≤ 20 cigarettes/day; 2 = > 20 cigarettes/day.Table 3 Hepatic microsomal enzyme activities (mean $\pm$ SD; n = 6) and liver/body weight ratios from control and rats exposed to petrol

	Control	Petrol exposed	% Increase
Aminopyrine N-demethylase*	74 $\pm$ 7	122 $\pm$ 11	65
Ethylmorphine N-demethylase*	75 $\pm$ 10	134 $\pm$ 21	79
Antipyrine metabolism†	35 $\pm$ 2	54 $\pm$ 5	54
Aniline hydroxylase‡	21 $\pm$ 1	33 $\pm$ 3	57
Benzo(a)pyrene hydroxylase§	42 $\pm$ 3	61 $\pm$ 8	45
Liver/body weight ratio	0.059 $\pm$ 0.001	0.067 $\pm$ 0.002	14

*nmol HCHO/min/g.

†nmol 14 C/min/g.

‡nmol PAP/min/g.

§Relative fluorescence units.

||All values significantly greater than control (p < 0.01).

0.33 mg/100 ml in controls and 0.28 ± 0.18 in exposed rats, and the levels for ALAT activity were 45.7 ± 11.3 IU/l in controls and 46.2 ± 12.0 IU/l in exposed rats.

Discussion

In this study antipyrine metabolism in a group of petrol station attendants was enhanced when compared with a group of subjects not occupationally exposed to petroleum. The implication of this finding is that one or more chemicals in the petrol station

environment is a microsomal enzyme inducer. Since petrol consists of a heterogeneous mixture of hydrocarbon molecules, with the number of carbon atoms ranging from four to 12 a molecule, it is an obvious candidate, although it is useless to speculate which compounds or types of compounds are the potential inducing agents in this mixture.

Previous studies in animals have shown the potential of petroleum to induce microsomal enzymes. Payne and Penrose¹⁸ found increased aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity in microsomal preparations of fish exposed to a 1 ppm emulsion of crude oil in water. Halogenated benzenes (compounds found in petrol) increased cytochrome P₄₅₀ content in the liver of rats given daily doses of 20 mg/kg po.¹⁹ No previous studies in man have shown petrol-induced microsomal enzyme activity. Although increased serum activity of glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, and ornithinecarbamoyl transferase have been reported in oil-tanker fleet workers,²⁰ it is questionable whether these changes reflect the activity of membrane-bound enzymes in the liver.

Rats exposed to petrol vapour during simulated working conditions over a period of 21 days were used to confirm that petrol might be the agent responsible for enhancement of antipyrine elimination in man. Induced in-vitro metabolism was found for all five of the cytochrome P₄₅₀ substrates tested. Hence the effect seems to be that of generalised induction of the microsomal mixed function oxidases.

Establishing that a correlation exists between antipyrine half life and petrol exposure would add weight to the hypothesis that petrol is the inductive agent. Attempts, however, to find an acceptable index of exposure to petrol proved unsuccessful. In previous studies phenol output in the urine has been used.²¹⁻²⁴ This method is based on the metabolism of benzene in the liver, producing phenol that is then conjugated and excreted. The major problem with this method is that the benzene content of petrol depends on the source of the crude oil from which the petrol is refined. In the study by Pandya *et al*²² the benzene content was quoted as 10-17% of petrol, but Sherwood²⁴ quoted 5%. In Adelaide the benzene content of petrol is about 3%; hence the sensitivity of the method would be substantially reduced. Although greater variability in phenol excretion was found in our sample of petrol station attendants, the amount excreted did not differ from that in the controls.

Since tetraethyl lead is added to petrol as an "anti-knock" agent, increases in body lead concentrations might be expected in petrol station attendants. Lob²⁵ found blood lead concentrations ranging from 0.80 to 2.95 $\mu\text{mol/l}$ (mean 1.30 $\mu\text{mol/l}$) in office workers and values of 1.11 to 2.85 $\mu\text{mol/l}$ (mean 1.84 $\mu\text{mol/l}$) in garage attendants. Moore *et al*²⁶ found that blood lead in petrol vendors was 1.58 ± 0.08 (SE) $\mu\text{mol/l}$, and this was greater than in a control population of public servants and university personnel, which was 0.69 ± 0.03 $\mu\text{mol/l}$. In the petrol-exposed subjects of the present study the mean value was less than that found for office workers in Lob's study, and the higher values were less than the upper 95% confidence limits expected in non-exposed workers.¹⁵ Thus blood lead concentrations did not differentiate the petrol station attendants in this study from the normal non-exposed population, nor did either ALAD activity in blood or urinary lead excretion, which were also within the normal range.

Although the metabolism of antipyrine has been found to be primarily under genetic control,²⁷ other factors, such as age, sex,²⁸ and smoking,²⁹ have been shown to alter its rate of metabolism. With this in mind, some personal and social measures were examined in the 19 petrol station attendants in an attempt to explain the difference in antipyrine metabolism, but no relation was found between antipyrine half life and age, work duration, smoking, or alcohol and caffeine intake. Furthermore, the distributions found for both smoking and caffeine intake were very close to those found in 307 healthy male North Americans.²⁸ This suggests that the drinking and smoking habits of the petrol-exposed group are not radically different from the general population and

that the altered metabolism of antipyrine cannot be attributed to differences in social habits.

Since antipyrine metabolism shows a two to three-fold variation in the general population, small changes in the activity of the enzymes that metabolise this compound are difficult to show when comparing two heterogeneous subgroups. This was illustrated in a study of antipyrine elimination in anaesthetists,⁵ where the inductive effect of trace levels of inhalational anaesthetics could be verified statistically only when intra-individual comparisons were made, but not when intergroup comparisons were made between anaesthetists and a control group. This might suggest that the inductive effect of exposure to petroleum is more substantial than that of exposure to traces of anaesthetic gases. The relation, however, between degree of shortening of antipyrine half life and the potency of the inducing agent is difficult to interpret because of the pronounced inter-individual variances. In the present study the decrease in antipyrine half life was 17%, and this is smaller than the 47% decrease seen in workers exposed to insecticides.³ In interpreting these changes, however, it must be borne in mind that in the latter study the mean half life of the control population was 13 hr compared with the 11.3 hr in the present study.

In conclusion the results indicate that petrol vapour is a moderately potent inducer of microsomal mixed function oxidases in rats, and that occupational exposure to petroleum may result in enhanced microsomal drug metabolism in man.

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