Effect of repeated exposure to aniline, nitrobenzene, and benzene on liver microsomal metabolism in the rat

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Wiśniewska-Knypl, Justyna M., Jabłońska, Janina K., and Piotrowski, Jerzy K. (1975). *British Journal of Industrial Medicine*, 32, 42-48. Effect of repeated exposure to aniline, nitrobenzene, and benzene on liver microsomal metabolism of the rat. Exposure of rats to aniline at daily doses of 50 mg/kg of body weight over a month stimulated the microsomal metabolism as manifested by (1) acceleration of p-hydroxylation of aniline and N-demethylation of aminopyrine in $9\cdot000 \times g$ postmitochondrial supernatant of the liver, (2) shortening the sleeping time after hexobarbital, and (3) reduction of the antipyretic effect of phenacetin.

In the rats exposed to nitrobenzene in a similar manner to aniline, nitroreduction of nitrobenzene and p-hydroxylation of aniline remained unaffected; the antipyretic effect of phenacetin was decreased, whereas hexobarbital sleeping time remained unchanged.

Exposure of rats to benzene (50 mg/kg of body weight daily for a month) had no effect on the rate of hydroxylation of benzene and N-demethylation of aminopyrine. In benzene-exposed rats hexobarbital sleeping time was prolonged whereas the antipyretic effect of phenacetin was unaffected.

Microsomal metabolism of aniline, nitrobenzene, and benzene was stimulated and inhibited when the rats were pretreated with phenobarbital and SKF 525-A, respectively.

The traditional approach to problems of industrial toxicology allows certain conclusions to be drawn based on controlled experiments in which a single dose of a poison is administered to the intact human and its metabolism studied. This is especially true for exposure tests which are further applied for men exposed daily in industry. This procedure is justified only if the compound in question is equally metabolized when administered once or repeatedly. The body burden as well as the excretion rate due to daily exposure may then be computed on a basis of kinetic data obtained from a single dose experiment (Piotrowski, 1971).

Metabolism of most foreign compounds is catalysed by reduced nicotinamide adenine dinucleotide phosphate (NADPH)—dependent micro-

somal enzymes in the liver (Gillette, 1966). Activity of the enzyme system may be stimulated unspecifically by a number of foreign compounds, and exposure to one compound can in turn lead to accelerated metabolism of others, applied later.

The assumption about the constant rate of metabolism of industrial poisons applied once and repeatedly needs to be verified in view of reports that stimulation of activity of microsomal enzymes may take place under the influence of a variety of drugs and chemicals (Conney and Burns, 1963).

This study was undertaken in order to test whether repeated exposure of rats to aniline, nitrobenzene, and benzene may finally lead to stimulation of the rate of microsomal metabolism of the compounds.

Materials and methods

Animals and treatments

Male albino rats of the Wistar strain, body weight 200-250 g, were used. The rats were caged in groups and were fed a commercial laboratory chow MURIGRAN and tap water ad libitum.

Aniline, nitrobenzene, and benzene in the prolonged experiments were given daily subcutaneously for a month at doses of 5 and 50 mg/kg of body weight. An emulsion in 0.1% aqueous Tween 80 was used for injection. In short duration tests rats were injected with the same substances at doses of 150 mg/kg of body weight daily for three days. The controls received 0.1% Tween 80.

The classical inducers of the drug-metabolizing microsomal enzymes, phenobarbital and 3,4-benzpyrene, as well as the inhibitor SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) (Conney and Burns, 1963; Gillette, 1966) have been used in the study as reference substances. Phenobarbital sodium in physiological saline was given intraperitoneally four times at 24-hour intervals in doses of 80 mg/kg of body weight according to Conney and Burns (1963). 3,4-Benzpyrene in olive oil was given according to Rickert and Fouts (1970) once intraperitoneally at a dose of 45 mg/kg 72 hours prior to an antipyretic test with phenacetin. SKF 525-A in physiological saline was given according to Kato, Chiesara, and Vassanelli (1962) once intraperitoneally at a dose of 50 mg/kg of body weight 30 minutes prior to (1) the killing of animals for *in vitro* assays of drug-metabolizing enzyme activity, or (2) before the administration of hexobarbital for the sleeping-time tests. In the case of antipyretic tests SKF 525-A was given in a dose of 25 mg/kg of body weight 45 minutes before phenacetin (Conney *et al.*, 1966).

Concurrent control groups to all test groups were handled in the same manner and injected with the proper vehicles, omitting the test-chemicals.

In vitro assays of liver drug-metabolizing enzymes

Rats were killed by decapitation. Liver was weighed, chilled on ice, and homogenized with 3 volumes of KCl (150 mmol)-Tris-HCl (20 mmol)-EDTA-Na (1 mmol) buffer, pH 7.4, in a glass Potter-Elvehjem homogenizer with a Perspex pestle. The homogenates were centrifuged at $9000 \times g$ for 15 minutes at 4°; the supernatants were aspirated and directly used for enzymatic assays.

Oxidative reactions were carried out in air and reductive ones under nitrogen. The $9000 \times g$ supernatant of the liver was used as the enzyme source for transformations of substrate and for generation of NADPH and it was supplemented with suitable substrates and cofactors. Incubation was performed at 37° in a Dubnoff-type metabolic shaker (100 oscillations per min). To insure adequate agitation of incubation medium two glass marbles were put into each flask.

The rate of aniline hydroxylation to p-aminophenol was measured for 15 minutes (Holtzman and Gillette, 1969) in a final volume of 3 ml suspension consisting of: Tris-HCl buffer, pH 7.4 (50 mmol), $MgCl_2$ (5 mmol), NADP (0.5 mmol), glucose 6-phosphate (10 mmol), aniline hydrochloride (1 mmol), and 1 ml of $9000 \times g$

tissue supernatant. Reaction was stopped by cooling the flasks on ice and, after extraction, p-aminophenol was determined colorimetrically by means of the indophenol reaction.

The rate of aminopyrine N-demethylation to yield formaldehyde was measured according to Dewaide and Henderson (1968) for 15 minutes. The reaction mixture in a final volume of 5 ml consisted of: Tris-HCl buffer pH 7.4 (50 mmol), semicarbazide hydrochloride (5 mmol), $MgCl_2$ (0.8 mmol), $MnCl_2$ (0.008 mmol), NADP (0.088 mmol), glucose 6-phosphate (5 mmol), aminopyrine (16.7 mmol), and 1 ml of the supernatant. Reaction was stopped by precipitation of protein with 1 ml of 25% $ZnSO_4$ and 1 ml of saturated $Ba(OH)_2$. The precipitate was removed by centrifugation and the amount of formaldehyde produced in the supernatant was determined according to Cochin and Axelrod (1959), a modification of the original Nash (1953) method.

The rate of benzene hydroxylation to phenol was measured for 30 minutes in a medium described by Lal, Puri, and Fuller (1970): the reaction mixture in a final volume of 8 ml consisted of sodium phosphate buffer, pH 7.4 (50 mmol), $MgCl_2$ (3 mmol), nicotinamide (25 mmol), NADP (0.16 mmol), glucose 6-phosphate (6.25 mmol), saturated level of benzene (99 mmol), and 2 ml of the supernatant. The reaction was stopped by cooling in an ice-bath. Steam-distilled phenol was determined colorimetrically with 2,6-dibromochinonochloroimide as described by Dutkiewicz, Piotrowski, and Kęsy-Dąbrowska (1964).

Reduction of nitrobenzene to aniline was assayed in a medium (Fouts and Brodie, 1957) containing sodium phosphate buffer, pH 7.4 (80 mmol), $MgCl_2$ (5 mmol), nicotinamide (20 mmol), NADP (0.12 mmol), glucose 6-phosphate (5 mmol), nitrobenzene (1 mmol; 5 μ l of 10% nitrobenzene in 98% ethanol, v/v), and 1 ml of the supernatant. The reaction was run for 15 minutes and stopped by cooling in an ice-bath. Protein was precipitated with 5% trichloroacetic acid, and in the clear supernatant aniline was determined after diazotization by coupling with 1-naphthol (Salm, 1958).

Protein was determined according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (fraction V) as a standard.

Student's *t* test was used for statistical evaluation of the results.

Indirect *in vivo* assay of microsomal metabolism rate

In order to evaluate the effect of the investigated substances on the rate of biotransformation in the intact organism additional unspecific tests were used. These were based on the duration of effects of standard drugs, which depends upon the activity of microsomal enzymes of liver cells.

(1) Hexobarbital sleeping time: Sleeping time was determined in rats injected with intraperitoneal 125 mg/kg body weight sodium hexobarbital in physiological saline according to Conney and Burns (1963). The time that had elapsed between losing and regaining the righting reflex was designated as sleeping time.

(2) Antipyretic activity of phenacetin: This test was performed on fevered rats according to Conney *et al.* (1966). The fever was induced by subcutaneous administration of 10 ml/kg body weight of 30% brewers'

yeast suspension in physiological saline. Phenacetin suspended in 2% gum acacia was administered by gavage at a dose of 200 mg/kg body weight 15 hours after the hyperthermia had been induced. Rectal temperature was taken hourly for four hours with an ELLAB Electric thermometer (ELLAB Instruments, Copenhagen).

Phenacetin in vivo is metabolized to pharmacologically inactive N-acetyl-p-aminophenol (Conney *et al.*, 1966). The pharmacological effectiveness of the former is decreased when the process is accelerated and vice versa. Thus, opposite effects are produced by 3,4-benzpyrene (inductor) and SKF 525-A (inhibitor).

Other compounds used in these two tests were administered as described above under "Animals and treatments".

Results

Aniline

The activity of microsomal enzymes after short-duration exposure and prolonged administration of aniline to rats is presented in Table 1. Prolonged as well as short-duration treatment of rats with aniline increased activity of aniline p-hydroxylase. An enhanced rate of N-demethylation of aminopyrine was observed only after one month's exposure to the higher dose of the compound (50 mg/kg body weight). It should be stressed, however, that the increment of activity of these enzymes was relatively scanty in comparison with the effect produced by phenobarbital.

Nitrobenzene

Attempts to reproduce in vitro the reaction of nitrobenzene hydroxylation to p-nitrophenol, which is considered the main metabolite both in humans and in rats, failed. Most probably the benzene ring was further hydroxylated to 4-nitrocatechol in the optimized in vitro system applied in this study.

Data concerning two other reactions involved in nitrobenzene metabolism are given in Table 2.

Exposure to nitrobenzene led to slight stimulation of activity of the microsomal enzymes. In the animals exposed for three days to the highest dose of nitrobenzene, 150 mg/kg of body weight, the activity of nitrobenzene nitroreductase was enhanced by about 50%. One month's exposure to 5 mg/kg of body weight daily of nitrobenzene led to 50% stimulation of activity of aniline p-hydroxylase. Phenobarbital, which was used in comparative tests, enhanced activity of the enzymes by about 100%.

Benzene

Neither the activity of benzene hydroxylase nor the activity of aminopyrine N-demethylase was modified by the treatment of rats with benzene (Table 3).

Benzene hydroxylase is an inducible enzyme: its activity in phenobarbital pretreated rats increased fourfold over the control value. On the other hand, SKF 525-A inhibited the hydroxylation of benzene.

Induction of the NADPH-dependent microsomal enzymes is usually accompanied by an increment of

TABLE 1
EFFECT OF PROLONGED EXPOSURE OF RATS TO ANILINE ON ACTIVITY OF DRUG-METABOLIZING ENZYMES IN 9000 × g SUPERNATANT OF LIVER: ANIMALS TREATED WITH PHENOBARBITAL AND SKF 525-A SERVED AS REFERENCE CONTROLS

Group	Treatment	Aniline p-hydroxylase (nmols of p-aminophenol per mg protein per 15 min ¹)	Aminopyrine N-demethylase (nmols of formaldehyde per mg protein per 15 min)
Control, 0.1% Tween 80 ..	1 ml/kg ^a daily for 3 days	1.7 ± 0.13 (6)	ND ^b
Aniline in 0.1% Tween 80 ..	150 mg/kg daily for 3 days	2.2 ± 0.08 ^a (6)	ND
Control, 0.1% Tween 80 ..	1 ml/kg daily for 30 days	1.9 ± 0.06 (12)	18.8 ± 0.7 (11)
Aniline in 0.1% Tween 80 ..	5 mg/kg daily for 30 days	2.3 ± 0.08 ^a (11)	19.7 ± 1.0 (10)
	50 mg/kg daily for 30 days	2.5 ± 0.10 ^a (12)	27.1 ± 1.5 ^a (11)
Control, 0.9% NaCl ..	1 ml/kg daily for 4 days	2.3 ± 0.12 (10)	ND
Phenobarbital in 0.9% NaCl ..	80 mg/kg daily for 4 days	5.0 ± 0.50 ^a (6)	ND
Control, 0.9% NaCl ..	1 ml/kg once	2.2 ± 0.05 (4)	ND
SKF 525-A in 0.9% NaCl ^a ..	50 mg/kg once	1.2 ± 0.02 ^a (4)	ND

¹Mean value ± standard error. Number of animals in each test group in parentheses.

^aSKF 525-A was injected intraperitoneally 30 min prior to killing the animals. In all other groups the last administration was 24 hours before killing.

^aSignificantly different from the control at $P = 0.01$ or 0.001 , respectively.

^bNot determined.

^cBody weight.

TABLE 2
ACTIVITY OF DRUG-METABOLIZING ENZYMES IN 9000 × g SUPERNATANT OF LIVER OF RATS WITH
PROLONGED EXPOSURE TO NITROBENZENE. RATS TREATED WITH PHENOBARBITAL AND SKF 525-A
WERE USED AS CONTROLS

Group	Treatment	Nitrobenzene reductase (nmols of aniline per mg protein per 15 min) ¹	Aniline <i>p</i> -hydroxylase (nmols of <i>p</i> -aminophenol per mg protein per 15 min)	Aminopyrine <i>N</i> -demethylase (nmols of formaldehyde per mg protein per 15 min)
Control, 0.1% Tween 80 ..	1 ml/kg ⁵ daily for 3 days	8.7 ± 0.8 (5)	1.8 ± 0.1 ¹ (5)	18.7 ± 0.5 ¹ (5)
Nitrobenzene in 0.1% Tween 80	150 mg/kg daily for 3 days	13.9 ± 2.0 ² (5)	1.9 ± 0.1 (5)	18.7 ± 0.8 (5)
Control, 0.1% Tween 80 ..	1 ml/kg daily for 30 days	7.4 ± 1.2 (7)	1.7 ± 0.1 (7)	13.7 ± 1.2 (7)
Nitrobenzene in 0.1% Tween 80	5 mg/kg daily for 30 days	10.5 ± 0.7 (7)	2.5 ± 0.1 ² (7)	17.7 ± 1.6 (7)
	50 mg/kg daily for 30 days	9.9 ± 0.8 (6)	2.0 ± 0.1 (7)	15.8 ± 1.6 (7)
Control, 0.9% NaCl ..	1 ml/kg daily for 4 days	7.5 ± 0.2 (5)	2.3 ± 0.1 (6)	14.5 ± 0.7 (5)
Phenobarbital in 0.9% NaCl ..	80 mg/kg daily for 4 days	15.4 ± 1.0 ² (5)	5.0 ± 0.1 ² (6)	21.6 ± 0.6 ² (5)
Control, 0.9% NaCl ..	1 ml/kg once	10.9 ± 0.85 ² (4)	ND ⁴	ND
SKF 525-A in 0.9% NaCl ..	50 mg/kg once	5.4 ± 0.70 ² (4)	ND	ND

¹Mean values are followed by standard error. Number of animals in each group in parentheses.

²Significantly different from the control at *P* = 0.05 and 0.01, respectively.

⁴Not determined.

⁵Body weight.

Other details as in Table 1.

microsomal protein content per whole liver and the increment of weight of the liver per 100 g of body weight (Conney and Burns, 1963). This was not the case in this study. The question arises how significant might be the in vitro recorded increments of the enzyme's activity for the in vivo rate of metabolism of foreign compounds. To solve this question the duration of hexobarbital narcosis and the antipyretic effect of phenacetin were measured

in animals that had been exposed for a month to aniline, nitrobenzene, and benzene at doses of 50 mg/kg of body weight/day.

Results of these indirect in vivo assays corroborated the results of the in vitro tests.

Hexobarbital sleeping time was shortened in the rats exposed to aniline (Table 4). It remained unchanged in the rats exposed to nitrobenzene, and it was prolonged in the benzene-exposed rats. The

TABLE 3
ACTIVITY OF DRUG-METABOLIZING ENZYMES IN 9000 × g SUPERNATANT OF LIVER OF RATS WITH
PROLONGED EXPOSURE TO BENZENE. RATS TREATED WITH PHENOBARBITAL AND SKF 525-A WERE
INCLUDED AS CONTROLS

Group	Treatment	Benzene hydroxylase (nmols of phenol per mg protein per 15 min) ¹	Aminopyrine <i>N</i> -demethylase (nmols of formaldehyde per mg protein per 15 min)
Control, 0.1% Tween 80 ..	1 ml/kg ⁵ daily for 3 days	3.4 ± 0.3 (6)	17.7 ± 1.3 (6)
Benzene in 0.1% Tween 80 ..	150 mg/kg daily for 3 days	3.9 ± 0.2 (6)	19.5 ± 2.1 (6)
Control, 0.1% Tween 80 ..	1 ml/kg daily for 30 days	3.6 ± 0.5 (6)	26.1 ± 2.3 (6)
Benzene in 0.1% Tween 80 ..	5 mg/kg daily for 30 days	3.7 ± 0.5 (6)	27.2 ± 1.3 (6)
	50 mg/kg daily for 30 days	4.3 ± 0.4 (6)	30.3 ± 2.9 (6)
Control, 0.9% NaCl ..	1 ml/kg daily for 4 days	4.4 ± 0.8 (6)	ND ⁴
Phenobarbital in 0.9% NaCl ..	80 mg/kg daily for 4 days	18.4 ± 2.7 ² (6)	ND
Control, 0.9% NaCl ..	1 ml/kg once	5.5 ± 0.2 (4)	ND
SKF 525-A in 0.9% NaCl ..	50 mg/kg once	2.8 ± 0.4 ² (4)	ND

¹Mean values are followed by the standard error. Number of animals in parentheses.

²Significantly different from the control at *P* = 0.05 and 0.01, respectively.

⁴Not determined.

Other details as in Table 1.

⁵Body weight.

TABLE 4

HEXOBARBITAL SLEEPING TIME OF RATS WITH PROLONGED EXPOSURE TO ANILINE, NITROBENZENE, AND BENZENE. RATS PRETREATED WITH PHENOBARBITAL AND SKF 525-A WERE INCLUDED AS CONTROLS

Group	Treatment	Sleeping time (minutes) ¹
Control, 0.1% Tween 80	1 ml/kg ⁵ daily for 30 days	25 ± 0.9 (5)
Aniline in 0.1% Tween 80	50 mg/kg daily for 30 days	20 ± 0.8 ² (5)
Nitrobenzene in 0.1% Tween 80	50 mg/kg daily for 30 days	23 ± 1.4 (5)
Benzene in 0.1% Tween 80	50 mg/kg daily for 30 days	30 ± 0.7 ² (5)
Control, 0.9% NaCl	1 ml/kg daily for 4 days	23 ± 0.9 (5)
Phenobarbital in 0.9% NaCl	80 mg/kg daily for 4 days	14 ± 1.0 ³ (5)
SKF 525-A in 0.9% NaCl ⁴	50 mg/kg once	320 (180-420) (3)

¹Mean values are followed by the standard error. Number of animals in each test group in parentheses.

^{2,3}Significantly different from the control at $P = 0.01$ or 0.001 respectively.

⁴SKF 525-A was injected 30 min prior to the dose of hexobarbital (125 mg/kg body weight) while the last dose of the remaining chemicals was given 24 hours earlier.

⁵Body weight.

inhibitor SKF-525-A and the inducer phenobarbital prolonged and shortened respectively the hexobarbital sleeping times.

The animals treated with aniline and nitrobenzene hardly ever reacted to phenacetin. The yeast provoked fever was only slightly decreased (Figure). Similar results were noted with the inducer 3,4-benzpyrene in the reference tests. SKF 525-A intensified the antipyretic effect of phenacetin.

Exposure of rats to benzene had no influence on the antipyretic activity of phenacetin.

Discussion

It has been reported that (1) rabbits exposed for 18 to 26 days to very low doses of aniline show an increased rate of excretion of p-aminophenol in the urine (Minkina, Lublina, Chekunova, and Dobrynina, 1970), and (2) aniline-adapted rats show an increased tolerance to injection of 300 mg/kg body weight of aniline (Lublina, Minkina, and Rylova, 1971). Both phenomena can be explained by the results of this study, namely, that repeated administration of aniline to rats leads to stimulation of the microsomal metabolism.

Benzene had no effect on the activity of the microsomal enzymes (Table 3) or on the antipyretic activity of phenacetin (Figure). Earlier Cornish, Wilson, and Abar (1970) reported that this compound at single doses of 100 mg per rat was inactive in respect of the microsomal hydroxylating system. Benzene may stimulate the microsomal metabolism in rats, mice, and rabbits but very high doses of about 1 to 2 g per kg of body weight must be used (Gonasun, Witmer, Kocsis, and Snyder, 1973). Nevertheless benzene at low doses of 50 mg/kg

body weight daily for 30 days caused significant prolongation of hexobarbital sleeping time (Table 4). This effect remains unexplained but it is just possible that benzene either inhibits the metabolism of hexobarbital or at this low dose of 50 mg/kg of body weight produces a narcotic effect that is additive to the narcotic effect of hexobarbital.

The activity of nitrobenzene as a potential stimulator of microsomal metabolism has not been experimentally checked yet.

This study indicates that nitrobenzene applied for a long time at low doses stimulates the activity of some microsomal enzymes (Table 2) and diminishes the antipyretic effect of phenacetin in yeast-fevered rats (Figure). The biological activity of nitrobenzene needs further study.

Have our results any significance for industrial toxicology? To answer this question we should compare the doses of the compounds applied in this study with threshold limit values of occupational exposure.

The maximal allowable doses are 35 mg, 35 mg, and 100 mg of aniline, nitrobenzene, and benzene, respectively, per man daily, i.e., 0.5–1.5 mg/kg of body weight (American Conference of Governmental Industrial Hygienists, 1972).

Rats are about 50 times less sensitive to aniline than man (Jenkins, Robinson, Gellatly, and Salmond, 1972) but there are no data on the basis of which we could say how tolerant rats are to nitrobenzene and benzene in comparison with man.

The aniline dose of 50 mg per kg body weight of a rat used in this study corresponds to an aniline dose of about 70 mg per man. Hence conclusions from this study may have some value for industrial toxicology.

FIGURE Effect of prolonged exposure of rats to aniline, nitrobenzene, and benzene (50 mg/kg body weight daily for 30 days) on the antipyretic activity of phenacetin. Animals treated with 3,4-benzpyrene and SKF 525-A served as the reference controls. Before the test a fever was evoked by subcutaneous injection of yeast suspension. Phenacetin was administered at 0 time. Groups untreated with phenacetin were included to ensure that (1) the temperature response to yeast suspension remained constant during the test time and (2) SKF 525-A alone had no antipyretic action at the dose used. Each group consisted of five rats. Vertical bars represent the standard deviation of the mean. For other details see the method described in the text.

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