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Further Investigations on the Evaluation of Exposure to Nitrobenzene

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Metabolic studies were performed on men exposed to nitrobenzene vapour under experimental conditions. Absorption was estimated by the analysis of urine for *p*-nitrophenol. About half as much vapour was absorbed through the skin as through the lungs.

In inhalation studies the accumulation of nitrobenzene in the course of repeated exposures was investigated. It was found that *p*-nitrophenol was excreted in the urine in increasing amounts on successive days of exposure, reaching, by the end of a week, levels approximately two and a half times as high as on the first day.

The specificity of the *p*-nitrophenol test on urine was studied using rats. Of all nitro-compounds investigated only chloronitrobenzenes, especially the *ortho*-isomer, gave interfering compounds in the urine. *o*-Chloronitrobenzene gave lower results than nitrobenzene by a factor of 3.

There are three important ways in which nitrobenzene may be absorbed under industrial conditions: as vapour through the lungs; as liquid through the skin; and as vapour through the skin. The first two processes have been investigated experimentally in man by methods which do not depend on knowledge of the metabolism of the compound in the body. Thus, Salmowa and Piotrowski (1960) measured the absorption of the liquid through the skin, and Salmowa, Piotrowski, and Neuhorn (1963) obtained data on the absorption of the vapour by the lungs.

Attempts have also been made to find an exposure test based on the metabolites of nitrobenzene. Salmowa and Piotrowski (1960) failed in using for this purpose the determination of *p*-aminophenol in urine; but Salmowa (1961) succeeded later in determining urinary *p*-nitrophenol both from animals and from man exposed to nitrobenzene. Salmowa *et al.* (1963) provided a quantitative interpretation of the *p*-nitrophenol test, as applied to man undergoing a single exposure by inhalation. This gave a basis for the studies in man of the skin absorption of nitrobenzene vapour which will be described in this paper.

The *p*-nitrophenol test, as described by Salmowa *et al.* (1963), could not be applied, however, to measure the absorbed quantities of nitrobenzene under industrial conditions, where the exposure is usually repeated daily. The test would apply only to the first day of exposure, whereas in the following days the levels of the metabolite could rise as an effect of accumulation. It was, therefore, necessary to study the kinetics of cumulation in experiments on man. This leads to a practicable test.

Before the general use of the test could be recommended, the specificity of the *p*-nitrophenol test had to be established, as in some factories nitrobenzene is produced in the same areas as other nitrocompounds which might produce metabolites interfering with the *p*-nitrophenol test. Specificity tests on rats were therefore carried out.

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Methods

Experiments on men were performed using an exposure chamber as described by Dutkiewicz (1960). The concentrations of nitrobenzene vapour (5 to 30 μg./l.) were obtained by the method of Salmowa *et al.* (1963).

Skin Absorption Studies Each subject was placed in the chamber in a hammock in such a way that practically the whole skin surface was exposed. They were supplied with fresh air from outside the chamber, so that no nitrobenzene could be absorbed through the lungs (see Dutkiewicz (1961), for empirical details). Concentrations of nitrobenzene vapour, humidity, and temperature were varied, and the effects of normal working clothes on absorption were also studied. The nitrobenzene absorbed through the skin was estimated from

urinary *p*-nitrophenol determinations using the method of calculation described by Salmowa *et al.* (1963).

Accumulation Studies For repeated exposure nitrobenzene was administered by inhalation (Salmowa *et al.*, 1963). The absorbed doses of nitrobenzene were estimated from measurements of the concentrations in the air, the volume of the expired air, and the mean retention, 80%, as found earlier. Four experimental subjects were used. One was exposed for six hours daily for four successive days. The remaining three were subjected to a longer exposure—the whole week (Monday to Saturday) and, after a pause on Sunday, again on Monday of the next week. In all experiments urine was collected quantitatively every few hours. *p*-Nitrophenol was determined in all specimens. For one subject urinary *p*-aminophenol was also determined.

Specificity Studies Specificity tests were carried out on groups of three rats for each compound, which was given by single subcutaneous injection as a freshly prepared suspension in ethanol/water (1/5, v/v). The rats were placed separately in glass metabolism cages ('Simax'). The doses, 40 μmole/kg., about 1 to 2 mg. per rat, were equivalent to more than 10 times the allowable level proposed for nitrobenzene in human exposure, *i.e.*, 25 mg. per person daily. The urine of the rats was collected over two days and the daily fractions were analysed separately for *p*-nitrophenol.

Nitrobenzene in Air Nitrobenzene in air was determined colorimetrically by the method of Piotrowski (1965a). Five litres of air were passed through an ice-cooled absorber containing 10 ml. water. Sodium dithionate (0·2 ml., 2·5% in 0·1 N NaOH) was added. After 10 min. the diazotizing solution was added (2 ml., 1·75% sodium nitrite in 1N HCl), and after a further 10 min. 2 ml. saturated potassium bicarbonate solution, 0·5 ml. freshly prepared saturated sulphamic acid solution, and 2 drops of R-salt (sodium 2-naphthol-3,6-disulphonate, 1% in water). The absorbance at 490 mμ was read after 30 minutes. The sensitivity was 3 μg., and the coefficient of deviation was 5%. As the efficiency of absorption of nitrobenzene in water was 91% results have been multiplied by 1·1.

p-Nitrophenol in urine was determined colorimetrically by the modification of the methods of Lawford and Harvey (1953) and of Vlachova (1956), described by Salmowa (1961). To 10 ml. of urine 2 ml. conc. HCl was added, and the mixture was heated to 100°C. for one hour to destroy conjugates. The mixture was then filtered, its *p*H adjusted to 10 by 40% NaOH, 0·5 ml. of hydrogen peroxide (30% in water) added, and the mixture held at 60°C. for 20 minutes. The *p*H was adjusted to 4 with conc. HCl, and, after cooling, the mixture was extracted twice with 25 ml. portions of solvent (light petroleum, b.p. 32-67°C., ether and *iso*-amyl alcohol, 4/1/0·05 by volume). The extracts were cleaned by shaking with oxalate buffer (2·5% oxalic acid and 2·5% dipotassium oxalate in water). The oxalate layer was removed and the *p*-nitrophenol was extracted from the light petroleum with 4 ml. 2N NH_3. The extract was reduced to *p*-aminophenol by adding 2 ml. conc. HCl and 1 g. zinc powder, and stirring for 3 minutes. The mixture was filtered, and the residue rinsed with 10 ml. of water. The aminophenol in the combined filtrates was converted to indophenol by the addition of phenol (1 ml., 5% in water) and conc. NH_3 (8 ml.). After 30 min. the absorbance at 630 mμ was measured. Standards were prepared by adding 5 to 100 μg. *p*-nitrophenol to 10 ml. specimens of urine from persons not exposed to nitro-compounds, and carrying out the procedure as above. The sensitivity was about 0·5 μg./ml. of urine, and the coefficient of deviation was 6%.

p-Aminophenol in urine was determined colorimetrically by a method based on the hydrolysis of a 10 ml. sample of urine with conc. HCl, dilution, and subsequent reaction with phenol and ammonia to form indophenol, as described by Piotrowski (1957). The sensitivity of the method was only about 10 μg./ml.

All colorimetric readings were carried out with a Coleman Jr. spectrophotometer and the Prism Absorptiometer Unicam SP-1400.

Results

Absorption of Nitrobenzene Vapour Through the Skin The results of exposure to different concentrations of nitrobenzene vapour of both naked and dressed men are shown in Table I. There was substantial absorption through the skin, which was proportional to the concentration of nitrobenzene in the air. Normal working clothes reduced the absorption by only 20 to 30%.

Table II shows the effects of varying humidity and temperature. Series 3 from Table I is included as a standard for comparison. In series 5, in which the temperature of the air was increased from 25° to 30°C., it was not possible for technical reasons to keep the humidity at 35% and it fell to 25%. Under these conditions the absorption of vapour was not increased. In higher humidity, however (series 6), the absorption was increased substantially, by about 50% on the average.

The maximum acceptable concentration (M.A.C.) is 5 μg./litre. From series 1 (Table I) a dressed person exposed for a full working day (six hours' exposure in Poland) would take up 7 mg. In addition he would take up about 18 mg./day via the lungs with a lung ventilation of 4 to 5 $m.^3$ and 80% retention. Thus the M.A.C. corresponds to a total uptake of 25 mg./day. This value is used in the following Sections.

Excretion of *p*-Nitrophenol in Subjects exposed Daily to Nitrobenzene In this experiment it was intended that the daily absorption of nitrobenzene should equal the maximum allowable dose, 25 mg. as calculated above. Due to the reduced lung ventilation of a man sitting and the

TABLE I

ABSORPTION OF NITROBENZENE VAPOUR THROUGH THE SKIN AT DIFFERENT CONCENTRATIONS

Series	*No. of Experiments*	*Nitrobenzene Concentration (μg./l.)*	*Absorbed Doses of Nitrobenzene (mg.)*	*Absorption Rate per Unit of Concentration (mg./hr.:μg./l.)*	*Notes*
1	3	5	11, 7, 4 (7)	0·23	Dressed
2	3	10	16, 14, 8 (13)	0·22	Dressed
3	3	10	19, 16, 10 (15)	0·25	Naked
4	3	30	80, 37, 46 (54)	0·30	Naked

Conditions were as follows: temperature 25°C.; humidity 35%; time of exposure, 6 hours. Mean values are shown in brackets.

TABLE II

EFFECTS OF TEMPERATURE AND HUMIDITY ON THE ABSORPTION OF NITROBENZENE VAPOUR THROUGH THE SKIN

Series	*No. of Experiments*	*Temperature (°C.)*	*Humidity (%)*	*Absorbed Doses of Nitrobenzene (mg.)*	*Absorption Rate per Unit of Concentration (mg./hr.:μg./l.)*
3	3	25	35	19, 16, 10 (15)	0·25
5	3	30	25	13, 16, 10 (13)	0·22
6	4	25	67	18, 34, 18, 24 (23)	0·38

Other conditions were as follows: air concentration, 10 μg./l.; persons naked; time of exposure, 6 hours. Mean values are shown in brackets.

exclusion of skin absorption, it was necessary to fix the vapour concentration at a higher level than the M.A.C. In the chosen concentration of 10 μg./litre, *i.e.*, twice the M.A.C., the absorption of nitrobenzene exclusively through the lungs for six hours daily (with a 0·5 hour pause in the middle of exposure) gave in individuals mean daily doses of 18·8, 18·2, 24·7, and 19·5 mg., the average volumes of inhaled air being in the range 2·2 to 2·9 m.^{3}.

The excretion curves of *p*-nitrophenol are shown in Figure 1. According to Salmowa *et al.* (1963), the total *p*-nitrophenol, m_∞, excreted from a dose of nitrobenzene can be calculated from the formula:—

$$m_\infty = m_t + V_t/0{\cdot}0115,$$

where m_t is the total recovered to the end of the experiment, and V_t is the average amount excreted per hour on the last day of the experiment. The last term in the equation corrects for the extra *p*-nitrophenol which would be excreted if sampling of urine was continued indefinitely. The corrections in these experiments are small. From the doses of nitrobenzene absorbed and the amounts of excretable *p*-nitrophenol, *i.e.*, m_∞, the percentage of nitrobenzene uptake excreted by each person as *p*-nitrophenol can be calculated. The percentages, mean value 16% (Table III), are in satisfactory agreement with an earlier value of 13% obtained after single exposures (Salmowa *et al.*, 1963).

Figure 1 shows that the excretion of *p*-nitrophenol rose for the first few days and became fairly steady after the third day. With the percentage conversions to *p*-nitrophenol calculated as above, by the fourth day the amounts excreted had substantially reached the maximum possible, *i.e.*, a steady state had been reached in which the *p*-nitrophenol excreted daily was, on the average, equivalent to 16% of the daily uptake of nitrobenzene. This finding is in accordance with the results of a detailed kinetic analysis of excretion after single (Salmowa *et al.*, 1963) and multiple (Piotrowski, 1965b) exposures.

Figure 2 shows the excretion rates of *p*-nitrophenol when the author took 5 mg. *p*-nitrophenol and 30 mg. nitrobenzene in two separate experiments. When *p*-nitrophenol itself was given, excre-

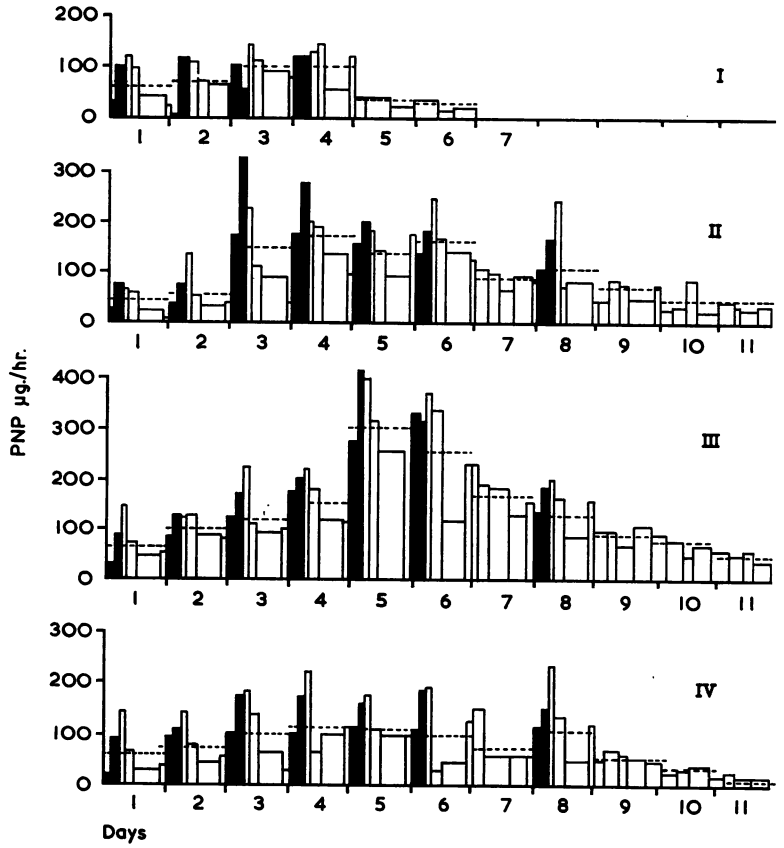

FIG. 1. Excretion rate of *p*-nitrophenol (µg./hr.) in urine from four men exposed daily to nitrobenzene.
(Shaded areas—excretion rate during exposure; dotted lines—average daily excretion rates.)

TABLE III

DAILY EXCRETION OF *p*-NITROPHENOL AS PERCENTAGES OF THE EQUILIBRIUM VALUES, CALCULATED FOR THE INDIVIDUAL SUBJECTS FROM THEIR INDIVIDUAL EFFICIENCIES OF CONVERSION

Subject	*Individual Conversion Efficiency (mole-%)*	*Excretion in Successive Days of Experiment*							
		1	2	3	4	5	6	7[1]	8
I	12	61	73	95	95	—	—	—	—
II	20	25	34	87	103	85	97	51	65
III	20	26	42	50	65	125	106	75	55
IV	13	44	61	83	92	89	78	61	89
Mean values	16	39	52	79	89	100	93	62	70

[1]No exposure.

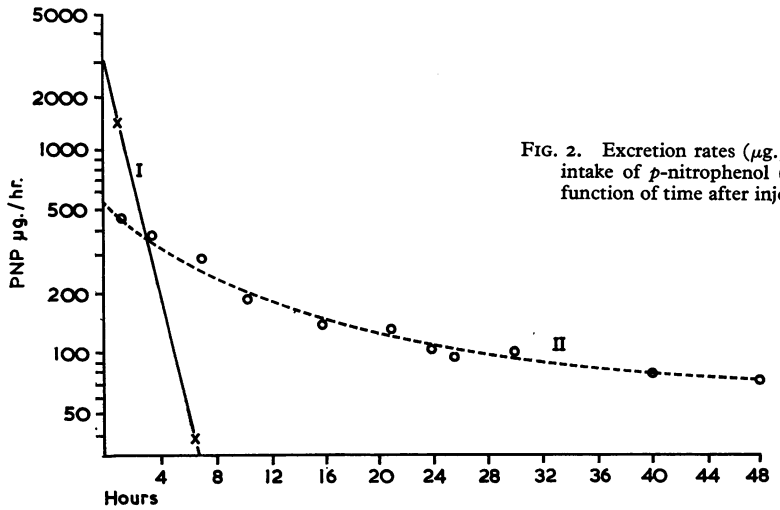

FIG. 2. Excretion rates (µg./hr.) of *p*-nitrophenol after oral intake of *p*-nitrophenol (I) and nitrobenzene (II), as a function of time after injection.

tion was very rapid, but when nitrobenzene was given, *p*-nitrophenol was only excreted slowly. It follows that the cumulation observed does not depend on the behaviour of the metabolite but is due to the slow rate of metabolism of nitrobenzene.

In the first, four-day, experiment on prolonged exposure to nitrobenzene *p*-aminophenol in urine was also assayed. None was found in any specimen.

Specificity of the *p*-Nitrophenol Test In rats treated as described under 'Methods', the dose of nitrobenzene administered produced concentrations of *p*-nitrophenol in the urine giving an absorbance of about 0·7 in the spectrophotometer. (The efficiency of conversion of nitrobenzene into *p*-nitrophenol was about 23%.) A compound was considered to give positive results if the absorbance exceeded 0·07, one-tenth of the above value.

Positive results were obtained only for *o*-chloronitrobenzene and 2,5-dichloronitrobenzene, for which the absorbances were 34% and 10% respectively of those found after an equimolar dose of nitrobenzene.

All other compounds tested gave negative results, *i.e.*, *m*-dinitrobenzene, *o*- and *p*-nitrotoluenes, 2,4-dinitrotoluene, 2,4,6-trinitrotoluene, *o*- and *p*-ethylnitrobenzenes, *p*-chloronitrobenzene, *m*- and *p*-nitroanilines, and 1-nitronaphthalene. All results refer to urine collected on the first day after injection. Urine collected on the second day gave negative results for all compounds including nitrobenzene itself.

Discussion and Conclusions

The data obtained from man give the following picture of the absorption of nitrobenzene and of the way in which it might be evaluated in industrial conditions. Nitrobenzene vapour is absorbed with 80% efficiency through the lungs and more slowly through the skin. The latter can, however, contribute appreciably to the total absorbed. The rate is much less than that of the absorption of liquid through the skin, which can reach about 2 mg./cm.^{2}/hr. (Salmowa and Piotrowski, 1960) and is the main source of danger in industry through the contamination with liquid of skin and clothing.

Absorption of vapour through the skin must, however, be considered in converting the M.A.C. in air to a maximum allowable dose. As shown under Results, the M.A.C. of 5 µg./l. corresponds to an uptake of about 25 mg./day, of which about a third may pass as vapour through the skin of even a clothed man.

The metabolism of nitrobenzene in man to *p*-nitrophenol is now well known and can be used as a test of exposure. *p*-Aminophenol, which has been claimed to be useful for this purpose, is undetectable in urine if the nitrobenzene absorption is low enough to be safe, even if the exposure to nitrobenzene is repeated daily. This does not contradict the finding of *p*-aminophenol by Ikeda and Kita (1964) in the urine of a patient poisoned with nitrobenzene, and by Piotrowski (1954) in workers exposed to high concentrations of vapour. The

subjects in both cases had absorbed far more than safe doses.

Man has been shown here to differ from rats in that he metabolizes nitrobenzene much more slowly, so that in man the concentrations of *p*-nitrophenol in the urine increased for about four days on repeated exposure, and the concentration on the first day was only about 40% of the peak value (Table III). If the daily exposure is substantially constant, then the amounts in the urine after the third day can be related by the data in Table III to the uptake of nitrobenzene. Three possible relationships are shown in Table IV. The marked superiority of the two methods in which the rate of excretion of *p*-nitrophenol is used over the method in which the concentration of *p*-nitrophenol in urine is used is very evident; the coefficient of deviation of the last is much higher. In practice the daily exposure fluctuates. A good estimate of the mean daily dose can, however, be obtained by taking urine specimens on each of the last three days of the working week, and this is the procedure recommended.

The *p*-nitrophenol test appears likely to be highly specific for nitrobenzene in practice. Only *o*-chloronitrobenzene gave interfering metabolites.

TABLE IV

TESTS FOR CONSTANT DAILY EXPOSURE TO NITROBENZENE, BASED ON THE EXPERIMENTAL VALUES OBTAINED IN THE LAST THREE DAYS OF THE WEEK, AND THE FACTOR BY WHICH EXCRETION RATES FROM FOUR TO SIX DAYS EXCEED EXCRETION RATES DURING THE FIRST DAY OF EXPOSURE

Unit of p-Nitrophenol Excretion	*Formula*	*Coefficient of Deviation (% of mean)*	*Factorial Increase over First Day*
Daily excretion (mg./day) ..	y = 0·18 z	33	2·6
In urine collected over last 3 hours of exposure (μg./ml.) ..	y = 0·21 z	69	2·1
Hourly excretion rate (μg./hr.)	y = 10·8 z	29	2·4

y = excretion of *p*-nitrophenol in the given units.
z = mean daily dose of absorbed nitrobenzene in mg.

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