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THE ACTIVITY OF ADENOSINETRIPHOSPHATASE, 5-NUCLEOTYDASE AND ALKALINE PHOSPHATASE IN BLOOD AND TISSUES IN GUINEA PIGS EXPOSED TO NITROGEN OXIDES

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The effect of long-term exposure of animals to nitrogen oxides at a concentration of 1 mg/m³ on the activity of ATP-ase, 5-nucleotydase and alkaline phosphatase in the blood, brain and liver was investigated. It was shown that a decrease in the activity of enzymes localized in cell membranes and involved in active transport was brought about by the toxic effect of nitrogen oxides. The elimination of magnesium, an activator of several membrane enzymes occurred as a consequence of the impairment of membranes which results from peroxidation of lipids of cell membranes. This phenomenon intensifies secondary disturbances in the activity of the studied enzymes. The binding of nitrogen oxides to gaseous ammonia leads to the formation of products which do not cause changes induced by nitrogen oxides only.

Nitrogen oxides enter into the organism through the respiratory tract. Their toxicity depends on the concentration and length of exposure; it increases in the presence of water vapour and metals which catalyze NO_2 oxidation resulting in the production of nitric acid. Acid aerosols formed in this way show a much stronger toxic effect than NO_2. Nitrogen oxides evoke the greatest changes in the respiratory system leading to the development of chronic bronchial catarrh and

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subsequent pulmonary emphysema. They penetrate into the alveoli, where they get absorbed, and then distributed into various tissues.

It is assumed that exposure to nitrogen oxide can also cause damage to other tissues. The oxidation of unsaturated fatty acids is one of the principal mechanisms of the toxic effect of nitrogen oxides (1—4). Free radicals formed during this process may react with other cell constituents or cytoplasmic membranes and lead to their impairment (5). These radicals react mainly with SH groups of proteins and enzymes (6). Under the influence of nitrogen oxides a cleavage of double links takes place (7). Since fats are among the main constituents of cell membranes we can suppose that their oxidation must lead to the impairment. This process should mainly affect the activity of enzymes localized in cell membranes. In order to trace this phenomenon the experiments were performed in animals exposed to nitrogen oxides.

Another series of experiments, in which nitrogen oxides were neutralized by gaseous ammonia was conducted to find out whether the observed changes are specific for nitrogen oxides.

MATERIALS AND METHODS

The experiments were performed in 90 male guinea pigs weighing from 307 to 358 g. The animals were bred in the Central Experimental Animal House of the Silesian Medical School and they were fed on a standard diet of green feed, oats and hay.

There were three groups of 30 animals each:

Group A. It consisted of guinea pigs who were kept in special chambers (1 m^3 capacity) and breathed fresh uncontaminated air. They served as controls.

Group B. This group was exposed to 1 mg/m^3 nitrogen oxides eight hours a day for a period of 120 days. The air in the chambers was exchanged eight times within each hour and the temperature was 21—22°C. During the experiment relative humidity ranged from 65 to 70%.

Group C. The third group of animals was used in experiments designed to anwer the question whether the changes observed are specific for the action of nitrogen oxides. The animals from this group were exposed to reaction products of nitrogen oxides and ammonia for 120 days, eight hours a day.

Nitrogen oxides were obtained in the following way: Small amounts of sulphuric acid were introduced through a feeder into the bulb containing a reserve of nitrite and potassium iodide solution. The reaction was:

$$2KNO_2 + 2\,KJ + H_2SO_4 \quad 2K_2SO_4 + J_2 + 2NO + 2H_2O$$

From a generator nitrogen oxide was forced through a set of protective elements so as to prevent iodide vapours or sulphuric acid cloud from

getting into the further parts of the apparatus. Pure nitrogen oxide thus produced was oxidized by oxygen at 120°C. The oxidation took place in the reactor constructed in the form of a 2 m log coil. The ratio of air to NO was 5:1 and secured a rapid oxidation according to the following reaction:

$$2NO + O_2 \rightarrow 2NO_2$$

The exposure air taken from the ambient atmosphere was passed through a filter made of flannel sacks absorbing particles of dust and of alkalized activated coal absorbing gaseous contaminants.

The dose was adjusted so as not to exceed nitrogen dioxide concentration in the air of the exposure chamber of 1 mg/m³.Ammonia in the amount slightly higher than that of the stoichiometric ratio was introduced into the stirrer in the form of 100% pure gas through a tortuous pump. It was mixed with the air containing nitrogen oxides at a volume concentration of 0.06—0.02%, and it was kept at 120°C for several seconds. A hot mixture of nitrogen oxides and ammonia was placed in the nozzle stirrer of the conduit supplying the exposure air into the chamber. A reaction of nitrogen oxides with ammonia took place in the conduit while the two compounds were mixed and cooled with the exposure air (Fig. 1).

The concentration of nitrogen oxides was checked with two independent methods:

a) continuously — by means of coulometric analyser of own design (8). The analyser was calibrated by a physical method using »permeation tubes« produced by »Dynacal« as well as tubes of own production;

b) periodically — samples of the exposure air were passed through bubbling washers and estimations were made according to *Saltzman's* method (9). A series of absorbing and oxidizing bubbling washers according to *Ellis* was used (10).

Ammonia concentration was checked periodically by *Nessler* method and determinations were made according to *Buck* and *Stratmann* (11)

Ammonia concentration was also checked continuously with the analyser of own design very similar in construction to the NO_2 analyser. The ammonia analyser was also calibrated by the »permeation tube« technique, with a »permeation tube« of own construction. The concentration of NO_2 and NO was measured daily. The mean values of the concentration throughout the exposure period amounted to: NO_2 = 1.04 mg/m³, NO =0.07 mg/m³.

The error calculated according to *Siekierzyńska* (12) was for NO_2 +10% and for NO — 10%. The mean ammonia concentration in the chambers over a period of four months was 0.78 mg/m³. During the reaction of nitrogen oxides with ammonia, the reaction products in the form of white dust were formed. The dust was deposited on the walls of the equipment. Dust particles were measured with a micrometric ocular of

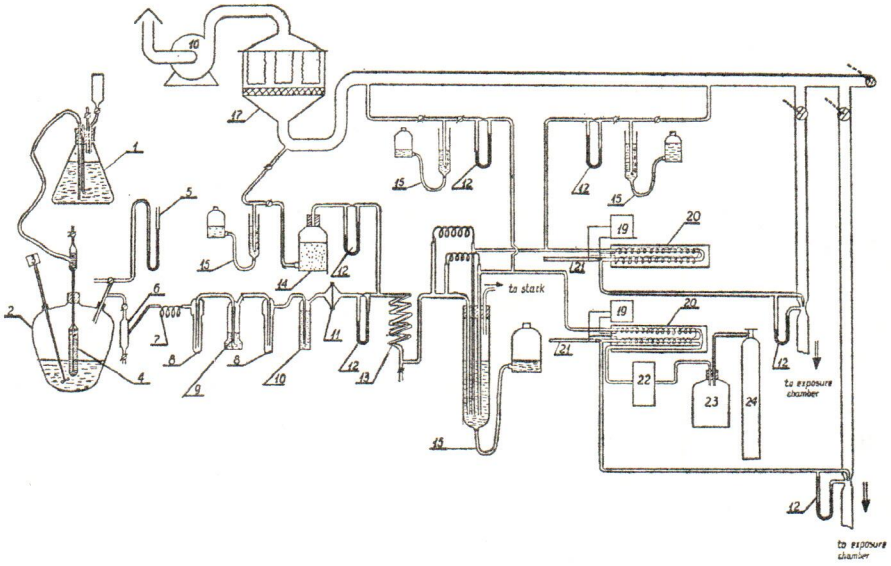

Fig. 1. *The equipment for the contamination of exposure air by NO_2 and NO_2 neutralized by ammonia.*

1. Sulphuric acid container — 2. Nitrogen oxide generator — 3. Stirrer — 4. Sulphuric acid feeder — 5. Manometer — 6. Dewaterer — 7. Resistance capillary tube — 8. Protection washers — 9. Washer containing sulphuric acid — 10. Dust filter containing raw wool — 11. Fibre glass paper filter — 12. Flow-meter — 13. Oxidizing reactor — 14. Air drier — 15. Manostat — 16. Ventilator — 17. Dust filter containing additionally activated coal — 18. Dose capillary — 19. Control autotransformer — 20. Heating furnace — 21. Thermometer — 22. Peristaltic pump — 23. Ammonia spare reservoir — 24. Large bottle containing ammonia.

total magnification 950 × (13). They were also analyzed chemically. The content of ammonia in the sample was estimated according to *Nessler* method (14) at the wave length of 340 nm.

The content of nitrites was determined according to *Griess* and *Trammsdorf* method with m-phenylediamine (14,15), and the content of nitrates with phenol-bisulphonic acid (14).

In all experimental and control animals examinations of behaviour were made and respiratory rate, heart rate and body weight were determined. In the peripheral blood the following parameters were measured:

1. the activity of ATP-ase according to *Jeanette* (16).
2. the activity of 5-nucleotydase by the method proposed by *Pletsch* and *Coffey* (17).
3. the activity of alkaline phosphatase according to *King Armstrong* method (18).

In urine samples collected daily from the animals which were kept in Roth metabolic cages the elimination of magnesium was checked with a Perkin-Elmer 403 atomic absorption spectrophotometer (17, 18). After 120 days of the experiment the animals were killed by exsanguination, and samples of the liver and brain were taken. Some of the collected samples were homogenized for two minutes with the piston revolving 3000 times/min, so that a complete grinding of tissues was obtained. Enzyme activities were determined in the homogenates of these tissues, and were expressed per 1 g of fresh tissue. Some of the samples were homogenized and mineralized, and the concentration of magnesium was estimated by means of a Perkin-Elmer 403 atomic absorption spectrophotometer (19,20). The results were calculated per 1 g of fresh tissue. A part of the tissues was fixed for 14 hours in cold Baker solution (+4 °C) and then cut on the freezing microtome into 10 μm sections. The reactions for adenosinetriphosphatase Ca-formol were made according to the method of *Wachstein-Meisel* (21). The incubation time at 37°C was 22, 40 and 45 minutes.

RESULTS

The animals exposed to nitrogen oxides showed an increased respiratory rate. They did not show any sign of excitement of aggressiveness and the heart rate did not increase. Manifold measurements of the size of the particles formed during the reaction of nitrogen oxides with ammonia showed the presence of only very small particles of dust. The size of the 74% of the particles was smaller than 1 μm, 23% ranged between 1—3 μm and only 3% was found to be between 3—4 μm. Repeated analyses of reaction products of nitrogen oxides and ammonia showed in 71% the presence of ammonium nitrate. The remaining am-

monia contained in specimens was bound in the form of $NH_4 NCO_3$ and $NH_2 COONH_4$ and its content amounted to 29%. The analytical error was 10%.

The animals of both experimental groups (B and C) survived the whole period of the experiment. The body weight of animals exposed to nitrogen oxides increased by 192 g in four months, while that of the animals to the reaction products amounted to 185 g. At the same time the body weight of the controls increased by 180 g. It follows that the exposure of animals to low concentrations of nitrogen oxides as well as to reaction products does not exert any significant effect on body weight.

Long-term exposure of animals to nitrogen oxides (B) brought about a decrease in the activity of adenosinetriphosphatase in the blood as well as in tissues, but the difference was statically significant only in tissues (Table 1). The activity of 5-nucleotydase (Table 2) was similar in all the groups. The activity of alkaline phosphatase (Table 3) was the lowest in group A. Generally, after the exposure of animals to nitrogen

Table 1

ATP-ase activity in blood serum, liver and brain homogenates. Each figure is a mean value (±SEX) and a range of the results obtained

ATP-ase activity in	Guinea pigs (N = 30) from group			Statistical difference
	A	B	C	
Serum (M-9 of P/ml/min)	4.13±0.69 (3.88—4.80)	4.10±1.31 (2.77—5.20)	4.06±0.66 (3.52—4.75)	A:B = N. S. B:C = N. S.
Liver (M-6 of/P/g/min)	65.8±5.59 (58.6—74.2)	57.0±1.77 (54.8—59.1)	62.7±4.44 (58.2—71.6)	A:B = P<0.001 B:C = P<0.001
Brain (M-6 of/P/g/min)	53.1±1.30 (50.7—55.3)	49.4±1.74 (47.7—52.0)	51.3±1.55 (48.8—52.6)	A:B = P<0.001 B:C = P<0.001

Table 2

The activity of 5-nucleotidase in blood serum, liver and brain homogenates expressed as means (±SEX) and as a range of the results obtained

5-nucleotidase activity in	Guinea pigs (N = 30) from group			Statistical difference
	A	B	C	
Serum (M-9 of P/ml/min)	5.07±3.30 3.87—7.20	4.42±3.49 (2.77—6.45)	4.70±2.39 (3.27—6.45)	A : B—N. S. B : C—N. S.
Liver (M-6 of/P/g/min)	3.06±1.20 (0.97—4.19)	2.62±1.39 (0.03—4.63)	2.97±0.63 1.94—3.61	A : B—N. S. B : C—N. S.
Brain (M-6 of/P/g/min)	0.93±0.36 (0.32—1.56)	0.68±0.44 (0.16—1.42)	0.68±0.21 (0.40—1.29)	A : B P<0.05 B : C—N. S.

Table 3

The activity of alkaline phosphatase in blood serum, liver and brain homogenates. The results are expressed as means (±SEX) and as a range

Alkaline phosphatase (in units)	Guinea pigs (N = 30) from group			
	A	B	C	Statistical difference
	13.8±3.4 (4.2—18.0)	7.6±2.7 (3.0—12.0)	12.7±1.6 (9.2—14.8)	A:B—p<0.001 B:C—p<0.001
	1108	857±146 (661—1200)	1220±115 (1100—1350)	A:B—p<0.001 B:C—p<0.001
	2682±651 1562—3525	2484±567 (1500—3225)	2852±536 (2062—4062)	A:B—N. S. B:C—p<0.02

Table 4

The level of magnesium in brain and liver and daily elimination with urine. The results are expressed as means (±SEX) and as a range

Specimen	Magnesium content in guinea pigs (N = 30) from group			
	A	B	C	Statistical difference
Brain μg/g	728.2±89.7 (690.0—910.0)	664.5±82.9 (490.0—7700)	788.0±73.2 (660.0—920.0	A : B—N. S. B : C p<0.001
Liver μg/g	600.5±57.0 (548.0—732.0)	434.3±23.8 (565.0—510)	632.5±53.9 (512—780)	A : B — p<0.001 B : C — p<0.001
Urine μg/day	416.0±202.1 (148.0—840.0)	3154.6±7773 (1630—6840)	740.8±261.2 (140.0—1132)	A : B p<0.001 B : C p<0.001

oxides, the enzyme activity measured in the blood as well as in tissues decreased. It was the same as in controls after the binding of nitrogen oxides to ammonia.

After the exposure to nitrogen oxides the concentration of magnesium in the brain and liver followed a decrease in the activity of the investigated enzymes. At the same time a marked elimination of magnesium with urine was observed. After the neutralization of nitrogen oxides by gaseous ammonia, the values of magnesium content in tissues were the same as in controls and the elimination of magnesium from the organism decreased.

Histoenzymic investigations of the brain in controls revealed a positive deffusive reaction for ATP-ase only on the walls of blood vessels and in the glia tissues (Fig. 2). After the exposure of animals to nitrogen oxi-

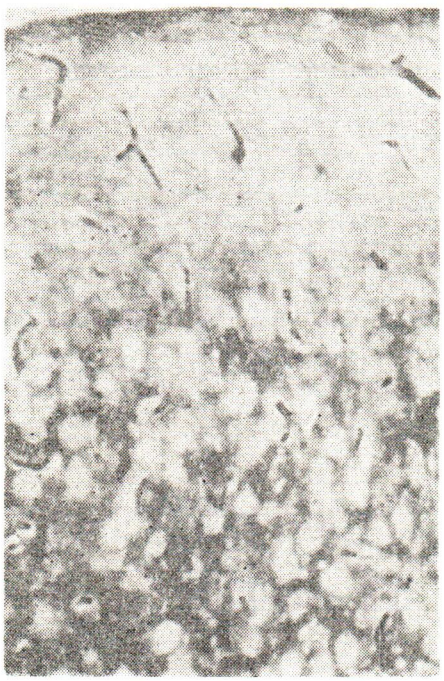

Fig. 2. *Diffusive enzymic reaction for ATP-ase in the wall of blood vessels in the brain of controls (Magn. 100x)*

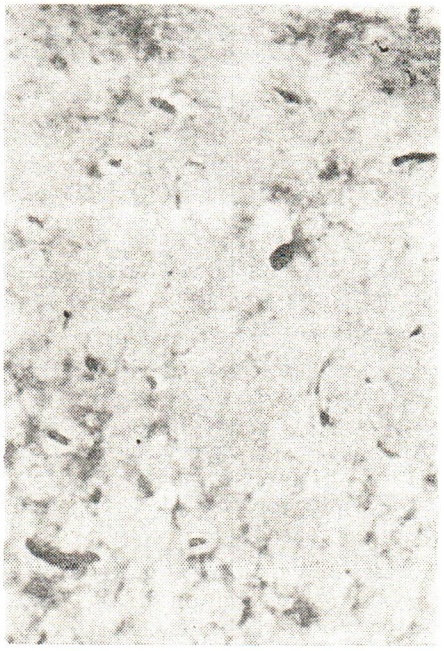

Fig. 3. *A slight weakening of the reaction for ATP-ase in the brain of animals exposed to nitrogen oxides (Magn. 100x)*

Fig. 4. *A positive reaction for ATP--ase in endothelia of peripheral vessels of lobule as well as in biliary tubules of the controls (Magn. 100x)*

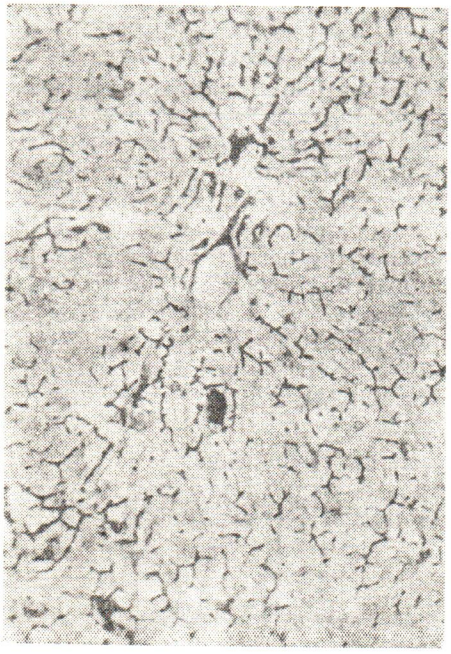

Fig. 5. *A sligt weakening of the reaction for ATP-ase in endothelia of peripheral vessels in animals exposed to nitrogen oxides (Magn. 100x)*

des a slightly decreased reaction in the glia tissue was observed (Fig.3). In animals from group C the reaction for this enzyme was similar to that of controls. A positive diffusive reaction was observed in the biliary tubules and in the endothelium of blood vessels of the lobule periphery and central veins (Fig. 4). After the exposure of animals to nitrogen oxides a lower enzyme activity in the endothelium of blood vessels and biliary tubules was observed (Fig. 5). The exposure of animals to reaction products (group C) did not produce any effect on the character, localization and intensity of the enzyme as compared with controls.

DISCUSSION

The results obtained indicate that nitrogen oxides cause lesions of cell membranes and lead to an increase in permeability and to the elimination of some metals necessary for the activity of several enzymes. Ellectrolyte displacements are probably due to the impaired functioning of cell membranes which results from the oxidation of unsaturated fatty acids and cholesterol. This may be reflected by the swelling of mitochondria and lysosome membranes which is observed in tissues after exposure to nitrogen oxides (1, 22). This process may affect the activity of enzymes localized in cell membranes. Using biochemical and histochemical methods I have demonstrated a decrease in the activity of ATP-ase, an SH enzyme which is a component part of the system responsible for active transport of sodium and potassium in cell membranes (23). A decrease in the activity of this enzyme may be caused either by the oxidation of SH groups under the influence of radicals formed by fat peroxidation in membranes or by the elimination of magnesium ATP-ase activator from tissues. Other enzymes localized in membranes showed a similar activity after the exposure to nitrogen oxides. Alkaline phosphatase activated by magnesium, and 5-nucleotydase showed a reduced time activity under the influence of nitrogen oxides. It may be assumed that the mechanism responsible for a decrease in the activity of these enzymes is similar to that of ATP-ase. The binding of nitrogen oxides to gaseous ammonia leads to the formation of products which do not cause the same changes as observed under the influence of nitrogen oxides only. *Goldstein* and co-workers (7) showed that activity of acetylcholinesterase localized in the membranes of erytrocytes decreased after the exposure to oxidizing compounds. At the same time a decrease in osmotic resistance of erythrocytes was observed (7).

These data suggest that other enzymes localized in membranes, which are necessary for the normal functioning of membranes, may also show changes of activity provoked by the action of nitrogen oxides.

CONCLUSIONS

1. Long-term exposure to nitrogen oxides brings about a change in the activity of enzymes involved in active transport as a result of peroxidation of lipids of cell membranes.

2. Impairment of membranes leads to secondary displacements of electrolytes, as shown for manganese, resulting in intense disturbances of enzymes localized in membranes.

3. The binding of nitrogen oxides to gaseous ammonia decreases considerably the disturbances produced by nitrogen oxides.

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Sažetak

AKTIVNOST ADENOZINTRIFOSFATAZE, 5-NUKLEOTIDAZE I ALKALNE FOSFATAZE U KRVI I ORGANIMA ZAMORČADI EKSPONIRANE DUŠIKOVIM OKSIDIMA

Istraživan je učinak dugotrajne ekspozicije životinja dušikovim oksidima u koncentraciji od 1 mg/m³ na aktivnost ATP-aze, 5-nukleotidaze i alkalne fosfataze u krvi, mozgu i jetri. U posebnim pokusima istraživan je učinak dušikovih oksida prethodno neutraliziranih amonijakom.

Utvrđeno je smanjenje aktivnosti enzima koji se nalaze u staničnim membranama i sudjeluju u aktivnom transportu. Primijećeno je, da se, kao posljedica oštećenja membrana, izazvanih peroksidacijom lipida u staničnim membranama izlučuje magnezij, koji je potreban za aktiviranje nekoliko membranskih enzima. Ova pojava pojačava sekundarne poremećaje u aktivnosti ovih enzima. Vezanje dušikovih oksida s amonijakom u plinovitom stanju dovodi do stvaranja produkata koji ne izazivaju promjene koje nastaju djelovanjem samih dušikovih oksida.

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