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THE INFLUENCE OF NITROGEN OXIDES AND PRODUCTS OF THEIR NEUTRALIZATION BY AMMONIA ON THE MYOCARDIUM AND BLOOD VESSELS

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The purpose of this work was to find out whether nitrogen oxides damage the myocardium and blood vessels, and if so to determine, the mechanism of these disturbances. Guinea pigs were exposed to the concentration of 1 mg/m³ of nitrogen oxides and to products of their neutralization with ammonia for a period of 120 days. Nitrogen oxides were found to reduce the activity of enzymes which take part in the electron and active transport in the myocardium. In this way they may increase myocardial ischemia evoked by other components of combustion gases. Nitrogen oxides disturb the metabolism of mucopolysaccharides and lead to their deposition in the aorta wall and to an increased elimination with urine. The neutralization of nitrogen oxides by ammonia prevents, to a great extent, the development of the changes in the myocardium and aorta wall.

Exhaust combustion gases formed by oxidation of solid, liquid and gaseous fuels are the main sources of atmospheric air pollutions. Carbon oxides, nitrogen oxides, sulphur oxides, lead oxides and hydrocarbons are some of the gaseous contaminants of the atmospheric air (1, 2). Exhaust combustion gases are known to exert a destructive effect on the walls of blood vessels and myocardium (3). This effect may be evoked by carbon monoxide, which can interfere with a group of oxidative enzymes, and produce disturbances in oxidation inside the cell. Anoxemia and damage of the myocardium result from carbon monoxide exposure (4, 5). Anoxemia also provokes disturbances in the metabolism of vascular walls. Nitrogen oxides may have a similar effect on oxygen metabolism of cells (6, 7). An increase of anaerobic processes

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resulting from the observed intensive glycogenolysis was found after the exposure to these gases (8). Approximately 90% of inhaled NO_2 is absorbed by the organism and can produce toxic effect on the lungs as well as on other organs. Oxidation of fatty acids of phospholipids is one of the principal mechanisms of the toxic effect of nitrogen oxides (9, 10). Phospholipids and cholesterol are among the components of cell membranes and their oxidation may cause disturbances in the permeability of biomembranes. The exposure to nitrogen oxides was found to cause electrolyte disturbances. So in the liver and brain the level of magnesium and zinc was markedly decreased, but at the same time their elimination with urine was considerably increased (10). The question is whether nitrogen oxides disturb oxygen metabolism of vascular wall tissues and cause a secondary damage to the myocardium. Besides, the purpose of this work was to find an answer to the question whether the binding of nitrogen oxides with gaseous ammonia reduces disturbances in the myocardium and aorta wall.

MATERIAL AND METHODS

The experiments were performed in 90 male guinea pigs ranging in weight from 315 to 420 g. During the experiment the animals were fed a standard diet of green feed, oats and hay.

The animals were divided into three groups of 30 guinea pigs each.

I. The control group consisted of 30 animals which were kept in toxicity chambers. The animals were allowed to breathe fresh uncontaminated air.

II. The animals from the second group were exposed eight hours a day for a period of 120 days to nitrogen oxides at a concentration of 1 mg/m³ in 1 m³ toxicity chambers. The mean concentrations for the whole period of repeated exposure were NO_2 0.94 mg/m³ and NO 0.07 mg/m³. The air in the chamber was exchanged 8 times an hour. During the experiment the temperature in the toxicity chamber was kept at 21—22° C, and relative humidity was between 65—70%. The methods of nitrogen oxide production as well as their estimation were published earlier (10).

III. Thirty animals of the third group were exposed to the reaction products of nitrogen oxides and ammonia eight hours a day for a period of 120 days. Nitrogen oxides were obtained in a specially constructed apparatus and then introduced into the reaction furnace at a concentration of 1 mg/m³. Ammonia at a concentration slightly higher than that of the stoichiometric ratio was also introduced. A reaction of nitrogen oxides with ammonia took place in the offtake during the process of mixing and cooling of the gases by purified atmospheric air. The reaction products were introduced into the chambers in which the animals were kept.

In all experimental animals as well as in controls the activity of phosphocreatine kinase (CPK) in blood serum was measured according to *Borkowski* (11) and the activity of asparagine aminotransferase (AsPAT) according to *Reitman* and *Frankel* (12).

In urine collected daily from the animals kept in Roth metabolic cages the concentration of nitrites and nitrates was estimated and calculated per milligram of creatinine. Besides the daily elimination of mucopolysaccharides in urine determined according to *Di Ferrante* (13), glycosaminoglycanes were determined by the turbidimetric method of the same author as described by *Mikolajczyk* (13).

After 120 days of experiment the animals were killed by exsanguination. After the autopsy the samples were taken from the heart and aorta for anatomopathological examinations. The samples were immersed in paraffin and stained with hematoxylin and eosin. The sample preparations were additionally stained after *Gieson* and *Mallory*. This allowed the differentiation between epithelial, connective, muscular and nervous elements. The staining with resorcine-fuxine after Weigert revealed elastic fibres. In the samples from the aorta the reactions for neutral mucopolysaccharides were made with the PAS method according to *McManus-Mowry*, and for acid mucopolysaccharides with alcyane blue after *Mowry*.

Samples from the heart and ascending aorta were also taken for histochemical investigations. The material was divided into two parts. One was immediately sectioned in the kryostat into 10 μm thick sections. The following reactions were made:

1. Succinic dehydrogenase (SC-D) according to *Pearse* (15) with tetra nitro BT produced by Sigma. Incubation time was six minutes in the heart and 67 minutes in the aorta at 37° C.
2. Isocitric dehydrogenase (SC-D) according to *Pearse* (15) with tetra nitro BT produced by Sigma. Incubation time was 14 minutes in the heart and 78 minutes in the aorta at 37° C
3. Lactic dehydrogenase (LD) according to *Pearse* (15) tetra nitro BT (Sigma). Incubation time at 37° C was 11 minutes in the heart and 22 minutes in the aorta.

The second part of the material was fixed for 14 hours in a cold (4° C) Baker's solution sectioned on freezing microtome into slices 10 μm thick and the reactions were made as follows:

1. $NADH_2$ — tetrazole reductase (NADH - TR) according to *Farber* (16). The incubation time at 37° C was 10 minutes in the heart and 20 minutes in the aorta.
2. Alkaline phosphatase (AP) according to *Gomori* (7) with betaglycerophosphate produced by BDH. The incubation period at 37° C was six minutes in the heart and 180 minutes in the aorta.

3. Adenosinotriphosphatase Ca-formol (ATP-ase) according to *Wachstein-Meisel* (17) with adenosinmono-phosphate produced by Koch-light. The incubation time at 37° C was 22 minutes in the heart and six minutes in the aorta.
4. Acid phosphatase (AP) according to *Gomori* (18) with betaglycerophosphate produced by BDA. The incubation time at 37° C was 20 minutes in the heart.
   The preparations were covered by glycerogel.

RESULTS

Repeated analyses of reaction products of nitrogen oxides with ammonia were described previously (19). A difference in the increase in body weight between the experimental and control groups was not observed. The activity of phosphocreatine kinase and asparagine aminotransferase in blood serum significantly increased in animals exposed to nitrogen oxides. After the exposure to reaction products the activity of these enzymes decreased (Table 1). In the course of exposure to nitrogen oxides the animals showed an increased elimination of nitrites, nitrates, mucopolysaccharides and glucosaminoglycanes. Nitrogen oxides bound by ammonia resulted in a decreased excretion of nitrites, nitrates, mucopolyccharides and glycosaminoglycanes.

Results of anatomopathological and histochemical investigations

Heart

The heart muscle as well as endocardium and epicardium did not show any pathological changes in controls (Fig. 1). After the exposure to nitrogen oxides hyperemia of the muscle was found in all samples and in nine samples focal fine-celled infiltrations mainly around the blood vessels were identified (Fig. 2). Fine-celled infiltrations were accompanied by a focal fragmentation of muscle fibres (Fig. 3). After the exposure to reaction products of nitrogen oxides with ammonia, the microscopic picture of the heart muscle was found to be normal in 28 samples out of 30 investigated (Fig. 4). Slight fine-celled infiltrations around the blood vessels of the heart muscle were found in only two cases.

Succinic dehydrogenase (SD)

A positive enzymatic reaction for SD was found in the sarcoplasm of muscle fibres in the control animals (Fig. 5). Fine granules of reaction products were arranged in a linear pattern along myofibrils. These granules were accompanied by a slightly marked diffuse component. Nuclei of syncytial cells were found to have no positive reaction.

Table 1.

The activity of phosphocreatine kinase (CPK) and asparagine aminotransferase (AsPAT) in blood serum of control animals, animals exposed to nitrogen oxides and those exposed to reaction products of nitrogen oxides with ammonia.

	Controls $\bar{x}_1 \pm SD$	Animals exposed to nitrogen oxides $\bar{x}_2 \pm SD$	Animals exposed to reaction products of nitrogen oxides with ammonia $\bar{x}_3 \pm SD$	Difference between $\bar{x}_1 - \bar{x}_2$	Probability level	Difference between $\bar{x}_2 - \bar{x}_3$	Probability level
CPK in internat. units according to Borkowski	219.55±41.3	375.4±111.2	170.13±82.9	155.85	<0,005	205.27	<0,001
AsPAT in units according to Reitman Frankel	32.9±7.76	53.8±13.64	38.9±10.16	21.1	<0,001	14.9	<0,001

Table 2.

Daily elimination of nitrates, nitrites, mucopolysaccharides and glycosaminoglycanes in the urine of control animals, animals exposed to nitrogen oxides and those exposed to reaction products of nitrogen oxides with ammonia

	Controls $\bar{x}_1 \pm SD$	Animals exposed to nitrogen oxides $\bar{x}_2 \pm SD$	Animals exposed to reaction products of nitrogen oxides with ammonia $\bar{x}_3 \pm SD$	Difference between $\bar{x}_1 - \bar{x}_2$	Statistical significance α	Difference between $\bar{x}_2 - \bar{x}_3$	Statistical significance α
Nitrates in urine after 1 month of exposure (in mg/mg creatinine)	0.080±0.010	0.280±0.077	0.141±0.017	0.20	<0.025	0.139	insignificant
Nitrates and nitrites in urine after 1 month of exposure (in mg/mg creatinine)	0.129±0.032	0.441±0.110	0.185±0.021	0.312	<0.02	0.256	<0.05
Mucopolysaccharides (in mg/day)	3.58±0.69	7.99±1.9	4.10±0.71	4,41	<0.001	3.89	<0.001
Glycosaminoglycones (mg/dm³)	12.0±1.7	19.6±4.9	13.2±1.8	7,6	<0.001	6.4	<0.01

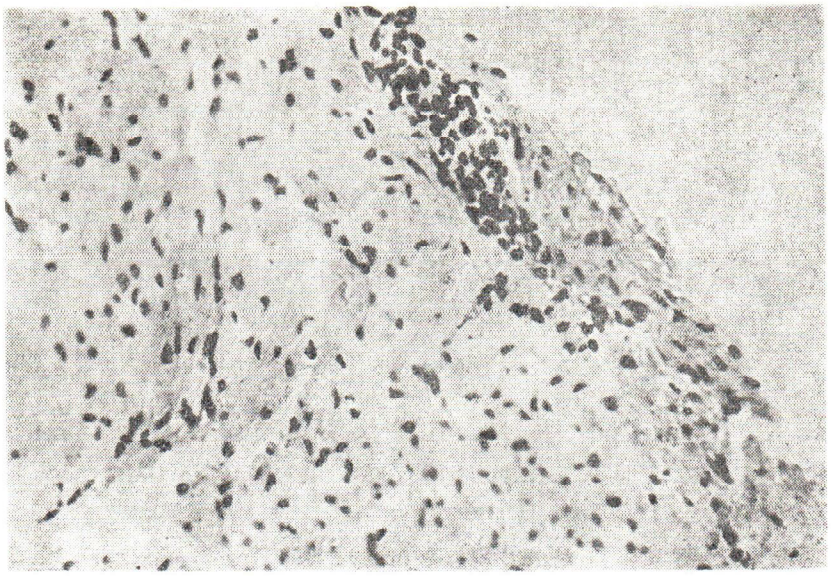

Fig. 2. *Fine-celled infiltrations in the heart muscle of animals exposed to nitrogen oxides. (hematoxylin-eosin, magn. ca. 400x)*

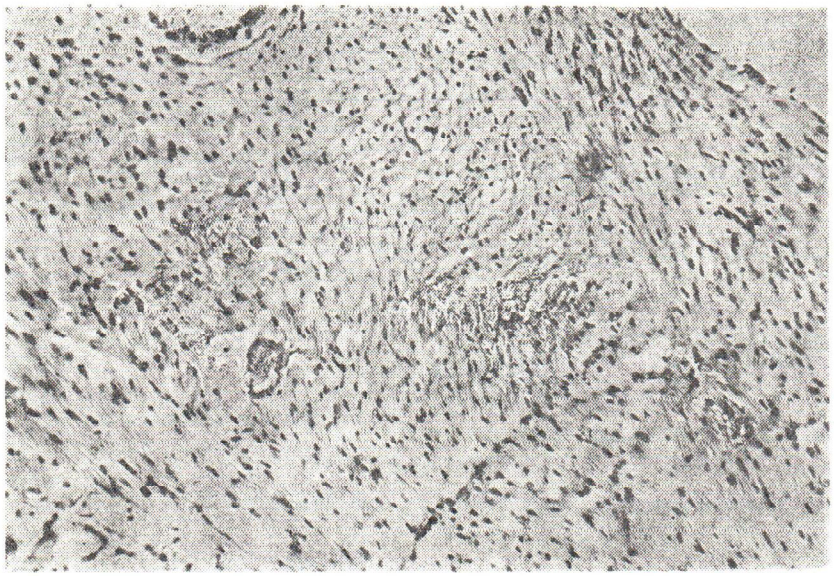

Fig. 1. *Normal structure of the heart muscle of control animals. (hematoxylin-eosin, magn. ca. 160x)*

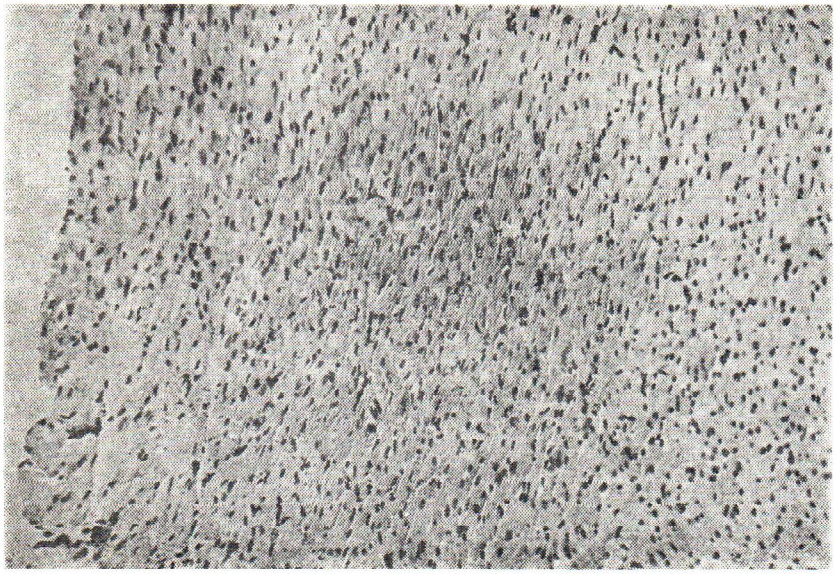

Fig. 4. *Normal structure of the heart muscle of animals exposed to reaction products of nitrogen oxides with ammonia (hematoxylin-eosin, magn. ca. 160x)*

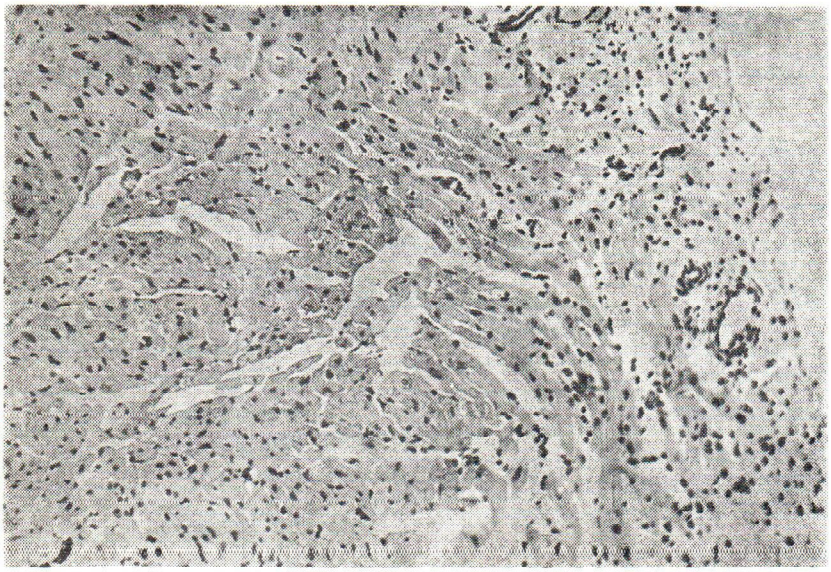

Fig. 3. *Fine-celled infiltrations and focal fragmentation of the heart muscle of animals exposed to nitrogen oxides. (hematoxylin-eosin, magn. ca. 160x)*

In animals exposed to nitrogen oxides the intensity of the reaction in particular muscle fibres diminished considerably (Fig. 6). Muscle fibres were characterized by an uneven distribution and intensity of enzymatic reaction — from weak to strong, from granular to diffuse.

The group of animals exposed to the reaction products of nitrogen oxides with ammonia showed a considerable increase in the reaction intensity as compared with the first group, but it did not reach the level of controls. However, a marked uneven distribution and intensity of the reaction was seen in a single muscle fibre as in the neighbouring ones.

Isocitric dehydrogenase

The histological findings in the control group were identical to those described for SD (Fig. 7).

After the exposure to NO_2 marked focal decrease of reaction intensity was found as compared with controls (Fig. 8). The character and localization of the reaction were similar to those observed in controls.

In the second group the reaction intensity was slightly increased with an uneven activity in particular fragments of muscle fibres. The character and localization of the reaction were similar to those in the control group.

Lactic dehydrogenase

The findings in the control group were the same as described for SD (Fig. 9). After the exposure to NO_2 the reaction intensity was distinctly decreased in most of the muscle fibres. The localization and character were as in controls (Fig. 10). In the second experimental group a slight increase of the reaction intensity was found as compared with group I. The character and localization were as in controls.

$NADH_2$ -tetrazol reductase ($NADH_2$ - TR)

The findings in controls were the same as described for SD (Fig. 11). After the exposure to NO_2 a marked decrease of reaction intensity was noticed in most of the muscle fibres of the heart wall (Fig. 12). The reaction appeared granular, and was accompanied by a very delicate diffuse component.

In the second experimental group the reaction intensity increased, but did not reach the level of controls. The character and localization of the reaction in the fibres remained unchanged.

Adenosintriphosphatase Ca-formol

In the control group a positive diffuse reaction was only found in the vascular network and in some elements of muscular membranes (Fig. 13). The vessels filled in with a positive reaction produced a well-filled network (Fig. 13).

Fig. 6. *A decrease of reaction intensity in muscle fibres for succinic dehydrogenase of animals exposed to nitrogen oxides (magn. ca. 400x)*

Fig. 5. *Positive enzymatic reaction for succinic dehydrogenase in the sarcoplasm of muscular fibres of control animals (magn. ca. 240x)*

Fig. 8. *A marked decrease of isocitric dehydrogenase activity in the sarcoplasm of muscle fibres of animals exposed to nitrogen oxides (magn. ca. 400x)*

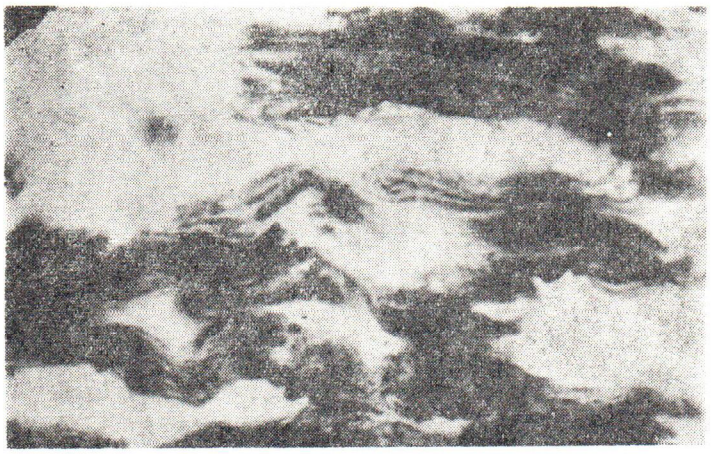

Fig. 7. *Fine grained reaction in the sarcoplasm of muscle fibres for isocitric dehydrogenase of control animals (magn. ca. 240x)*

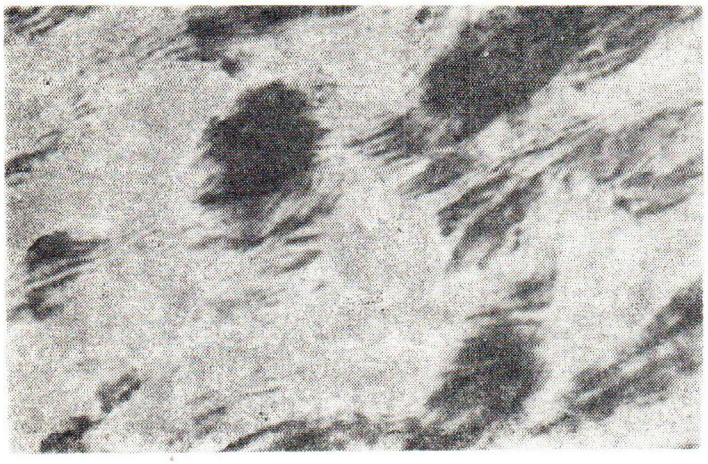

Fig. 10. *A drop of lactic dehydrogenase activity in most of muscle fibres of animals exposed to nitrogen oxides (magn. ca. 400x)*

Fig. 9. *Fine-grained diffuse reaction along muscle fibres for lactic dehydrogenase of control animals (magn. ca. 400x)*

Fig. 12. *A significant decrease of reaction for $NADH_2$ tetrazol reductase of animals exposed to nitrogen oxides. It concerns most fibres of the heart muscle (magn. ca. 240x)*

Fig. 11. *Fine-grained diffuse reaction for $NADH_2$ tetrazol reductase along muscle fibres of control animals (magn. ca. 240x)*

After the exposure to NO_2 the reaction intensity for this specific phosphatase was greatly reduced in all blood vessels (Fig. 14). The vessels no longer formed a regular spatial network.

In the second group enzymatic reaction returned to the level of controls.

Alkaline phosphatase

The reaction for this enzyme in experimental groups was the same as in controls.

Acid phosphatase

In the control group a positive enzymatic reaction was found both in the sarcoplasm of the muscle fibres (Fig. 15) as well as in connective cells. In the sarcoplasm the reaction was granular, very weak and its localization was comparatively uneven, either under sarcolema or more towards the center. After the exposure to NO_2 the reaction intensity in muscle fibres was slightly increased giving rise to a greater number of granulations with AP activity in the sarcoplasm (Fig. 16) as well as to a greater number of phagocytes of connective tissue origin.

The findings in the second experimental group was the same as in controls.

Aorta

Internal and median membranes and adventitia of control animals did not show any pathological changes.

After the exposure to nitrogen oxides, mucopolysaccharides content in the wall of the aorta increased in 15 cases; acid mucopolysaccharides were specially abundant in the aortic walls. In the remaining samples the increase of mucopolysaccharides was slight.

In all cases the aortic wall did not show any pathological changes after the exposure to the reaction products of nitrogen oxides with ammonia. No differences in the activity of succinic dehydrogenase, isocitric dehydrogenase, $NADH_2$ — tetrazol reductase and lactic dehydrogenase in the aorta wall were found.

DISCUSSION

The investigations performed demonstrated that nitrogen oxides affect the activity of several enzymes in the myocardium. The exposure to nitrogen oxides leads to a decreased activity of the enzymes which take part in the electrontransport i. e. in oxygen metabolism. The mechanism of this phenomenon is unknown. *Ramazotto* and coworkers (7) showed, that the in vitro exposure of the lung, liver and kidney homogenates to nitrogen oxides reduces drastically the activity of cytochrome oxidase and succinic dehydrogenase. A decreased activity of these enzymes may result from a drop of pH.

Fig. 14. *A marked decrease of reaction for ATP-ase in all blood vessels of animals exposed to nitrogen oxides (magn. ca. 100x)*

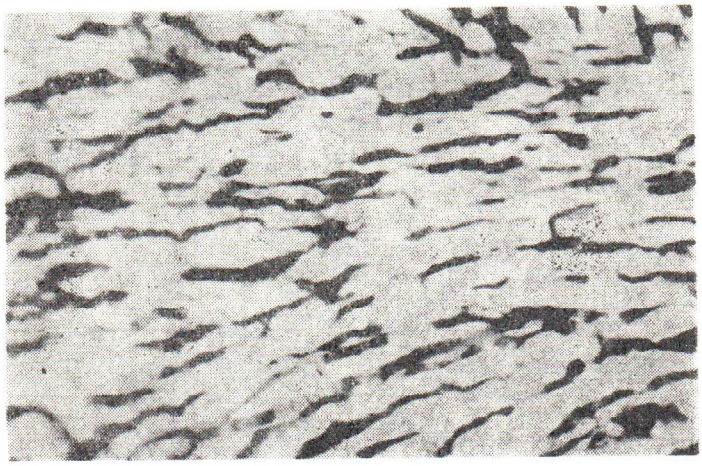

Fig. 13. *Positive diffuse reaction for ATP-ase in vascular reticulum and in some elements of muscle membranes of control animals (magn. ca. 100x)*

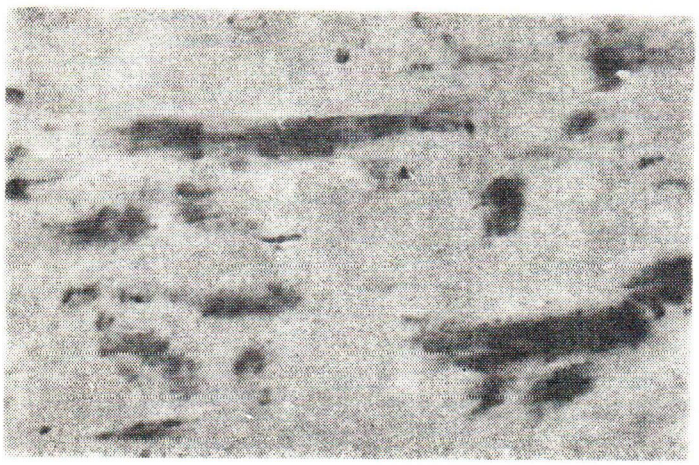

Fig. 16. *Increased activity of the reaction for acid phosphatase both in the sarcoplasm and in phagocytes (magn. ca. 240x)*

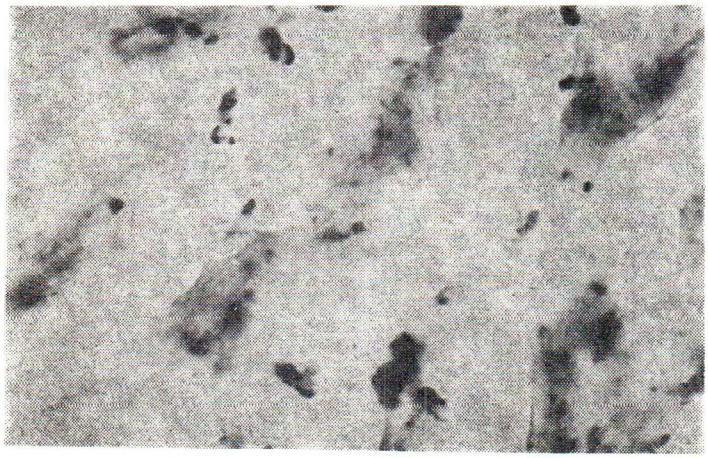

Fig. 15. *Granular reaction for acid phosphatase in the sarcoplasm of muscle fibres of control animals (magn. ca. 400x)*

Nitrogen oxides are neutralized in organism by buffer systems of blood, and are eliminated from organism with urine in the form of nitrates and nitrites. An increased elimination of these compounds with urine after the exposure to nitrogen oxides was observed in this experiment. In animals exposed to nitrogen oxides a metabolic acidosis was compensated by respiratory alcalosis (8). It resulted from the respiratory absorption of acid components, i. e. nitrogen oxides. Nitrogen oxides disturb not only energetic processes in the cells but also influence the activity of cell membranes and their permeability. A displacement of ions results from lipid oxidation of biomembranes. At the same time enzyme activity in the transport of active ions decreased. ATP-ase activity was reduced while acid phosphatase activity in the myocardium was increased. This may indicate an increase of catabolic processes. The anatomopathological investigations revealed fine-celled infiltrations in the myocardium mainly around the blood vessels and focal fragmentation of muscle fibres. The question remains whether the observed changes are due to the damage of blood vessels or nitrogen oxides exert a direct toxic effect on the myocardium.

In performed investigations we noticed no changes in the activity of enzymes which take part in both electron and active transport in the aorta wall. It was very interesting to find a marked increase of mucopolysaccharides, specially acid mucopolysaccharides in the wall of the aorta. At the same time a higher elimination of mucopolysaccharides and glycosaminoglycanes with urine was observed. Several observations indicate that nitrogen oxides disturb the metabolism of carbohydrates (6, 8, 20) and activate glycolysis. The observed increase of glycolytic enzyme activity can result from the adaptation of organism to the disturbances oxidation processes. The exposure to nitrogen oxides brings about disturbances in the metabolism of mucopolysaccharides. These changes influence the metabolism of connective tissue, particular by its basic amorphic substance. These data could point to the disturbances of the metabolism of mucopolysaccharides in the vascular wall as well as of oxygen metabolism in the myocardium. Nitrogen oxides can react synergetically with carbon monoxide and lead to myocardial ischemia. Neutralization of nitrogen oxides by ammonia prevents, to a great extent, the development of the changes both in the myocardium and aorta wall.

CONCLUSIONS

1. Nitrogen oxides decrease the activity of enzymes which take part in the electron and active transport in the myocardium and in this way increase myocardial ischemia evoked by other components of combustion gases.
2. Nitrogen oxides disturb the metabolism of mucopolysaccharides and lead to their deposition in the aorta wall and to increased elimination with urine.

3. Neutralization of nitrogen oxides by ammonia prevents, to a great extent, the development of the changes both in the myocardium and aorta wall.

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Sažetak

UTJECAJ DUŠIKOVIH OKSIDA I PRODUKATA NJIHOVE NEUTRALIZACIJE S AMONIJAKOM NA MIOKARD I KRVNE ŽILE

Ova su istraživanja provedena sa svrhom da se utvrdi da li dušikovi oksidi uzrokuju ikakve poremećaje strukture miokarda i krvnih žila odnosno da li su poremećeni oni enzimski sustavi koji su važni za njihovo pravilno funkcioniranje.

Istraživanja su obavljena na zamorčadi od po 30 životinja. Jedna je skupina služila kao kontrola i udisala svježi zrak, druga je bila eksponirana dušikovom oksidu u koncentraciji od 1 mg/m³ 8 sati dnevno tijekom 120 dana a treća kroz jednako vrijeme produktima reakcije dušikovih oksida i amonijaka. U svih je životinja nakon 120 dana ekspozicije u serumu mjerena aktivnost kreatininfosfokinaze i asparagin aminotransferaze.

U životinja eksponiranih dušikovu oksidu, aktivnost spomenuta dva enzima bila je povećana u usporedbi sa kontrolnim životinjama. U istih je životinja utvrđeno i povećano izlučivanje nitrita i nitrata, mukopolisaharida i glikozaminoglikana u mokraći, a to upućuje na poremećeni metabolizam mukopolisaharida i potpomaže njihovu odlaganju u stijenci aorte.

Histokemijskim tehnikama mjerena je aktivnost sukcinilne, izocitrične i laktatne dehidrogenaze, te $NADH_2$-tetrazol reduktaze, alkalne i kisele fosfataze i adenozintrifosfataze. Rezultati histološkog i histokemijskog ispitivanja miokarda i aortalne stijenke upućuju na to da dušikovi oksidi smanjuju aktivnost enzima koji su potrebni u transportu elektrona u miokardu što bi moglo pripomoći povećanju ishemije miokarda što je izazvana različitim komponentama u plinovima nastalim sagorijevanjem.

Neutralizacija dušikovih oksida amonijakom smanjuje u velikoj mjeri nastajanje spomenutih promjena u miokardu i stijenci aorte.

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