

Arh. hig. rada, 29 (1978) 3.

THE EFFECT OF NITROGEN OXIDES ON
CARBOHYDRATE METABOLISM

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(Received for publication December 2, 1976)

The effect of nitrogen oxides on some parameters of carbohydrate metabolism in guinea pigs was studied. The exposure to nitrogen oxides decreases the activity of the enzymes which take part in the oxygen transport and simultaneously it increases the activity of the enzymes of glycolytic metabolism. Nitrogen oxides enhance the secretion of catecholamines and in this way they may increase glycolysis and glucose level in the blood. Exposure to nitrogen oxides disturbs the metabolism of glycoproteins and mucopolysaccharides.

Nitrogen oxides are inhaled through the respiratory tract together with the atmospheric air. Approximately 90% of inhaled nitrogen dioxide is absorbed into the organism (1). Nitrogen oxides react with water and produce nitric and nitric acids. In the presence of a buffer system the acids are neutralized. These compounds are eliminated from the organism with urine in the form of nitrites and nitrates (2). In animals exposed to nitrogen oxides metabolic acidosis was found. This was manifested by a defect in the buffer ability of the plasma compensated by respiratory alkalosis. During acidosis several metabolic disturbances were observed, one of them being a disturbance of carbohydrate metabolism. For this reason we tried to evaluate the effect of nitrogen oxides on some parameters of carbohydrate metabolism. Our purpose was

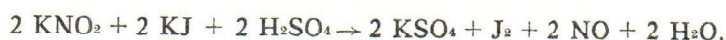
This work was supported by Environmental Protection Agency U. S. A. Contract No 5-533-1.

to demonstrate the disturbances of carbohydrate metabolism after exposure of animals to nitrogen oxides and to explain the mechanism of these disturbances.

MATERIALS AND METHODS

The investigation was carried out on 60 male guinea pigs ranging in weight from 430—705 g. During the experiment the animals were fed a diet of green feed, oats and hay. They were divided into two groups:

The control group consisted of 30 guinea pigs which were kept in toxicity chambers and breathed fresh air. Thirty animals of the experimental group were exposed to 1 mg/m³ nitrogen oxides in inhalation chambers (1 m³ capacity) eight hours a day for a period of 122 days. The air in the chamber was exchanged eight times an hour. The temperature in the toxicity chamber was kept at 21° to 22° C, and relative humidity at 65 to 70% during the experiment. Nitrogen oxides were generated in a specially constructed apparatus. Nitrogen oxide was formed according to the following reaction:



The system was self-regulating with steady nitrogen oxide overpressure in the generator. Nitrogen oxide was mixed with purified atmospheric air in the oxidizing reactor in ratio 1:5 where it oxidized to nitrogen dioxide.



The installation was described in a previous work (3). The concentration of nitrogen oxides in exposure chambers was checked by two independent methods:

- a) continuously — by a coulometric analyser of own design (4)
- b) periodically the samples taken from the exposed air were passed through bubbling washers and the estimations were made according to *Saltzman's* method (5).

In all experimental and control animals the following parameters were investigated: behaviour, frequency of respiratory rate, heart beat and body weight. The following measurements were carried out in blood serum 122 days after the beginning of the experiment.

1. glucose level by orthotoluidine method.
2. the activity of aldolase 1,6 — diphosphofructose by *Bruns-Puls* method (6)
3. the activity of lactic dehydrogenase (L. D. H.) according to *Cabaud-Wroblewski* (7)
4. the activity of phosphohexoisomerase (PHI) according to *Orlowski* (8)

5. the activity of sorbitol dehydrogenase (SDH) by the method of *Sewell* and co-workers according to *Kokot* (9)
6. the activity of isocitrate dehydrogenase (IDH) according to *Kokot* (9)
7. lactic acid level according to *Barker* and *Summers* (10)
8. seromucoid level in mg % of tyrosine according to *Winzler* (11)
9. the level of hexosamine binding by glycoproteids in mg% according to the method of *Elson* and *Morgan* modified by *Rimington* (12)
10. the level of sialic acid binding by glycoprotein proteins in mg % by the resorcinol method according to *Rzepecki* (13)

In urine samples collected from the animals kept in Roth metabolic cages the following parameters were estimated: pH and glucose content, the elimination of mucopolysaccharides with urine by *Di Ferrante* method (14) and the elimination of 3 methoxy - 4 hydroxyamylgadic acid (metabolite of catecholamines).

After 122 days of the experiment the animals were killed by exsanguination. After the autopsy the samples from the liver and brain were taken for biochemical investigations. The samples were washed in cold saline, dried between two drains of filter paper and placed in a freezer for 30 minutes. The sample weighing 4 g was homogenized in a Potter-Elvehjem homogenizer in the glass teflon system (rotation of the piston 3000/min). Diluted homogenate (2:8) was cooled to 0° C with double distilled water for two minutes. In this way the tissues are completely homogenized.

The homogenates were filtered through gauze and centrifuged for 10 minutes. The activities of the same enzymes were determined in the homogenates as in the serum. They were calculated per 1 g of fresh tissue. Besides, in the liver homogenates the contents of hexoses (11), hexosamines (12), sialic acids binding by proteins, glycoproteids according to *Warren* (15), and seromucoid according to *Winzler* (11) were estimated and expressed in mg per 100 g of fresh tissue.

Samples from the liver and brain (frontal lobes) were taken for histochemical investigations. They were divided into two parts. One was immediately sectioned in a kryostat into 10 μ m thick sections and the histochemical reactions were made for succinic dehydrogenase (SD) according to *Pearse* (16) by means of tetra nitro BT produced by Sigma. Incubation time (at 37° C) was eight minutes in the liver and 54 minutes in the brain. The rest of the samples were fixed for 14 hours in cold (+ 4 °C) Baker's solution. They were sectioned with a freezing microtome into 10 μ thick slices and the reactions were made for NADH₂ — (tetrazole) reductase according to *Farber* (17).

The incubation time (at 37 °C) was 35 minutes for the liver and 32 minutes for the brain. The preparations were covered with glycerogel.

RESULTS

The animals exposed for a long time to nitrogen oxides showed an increased respiratory rate. The body weight of the animals exposed to nitrogen oxides increased by 192 g during four months. At the same time the body weight of the control group increased by 190 g. It appears accordingly that the exposure of animals to a low concentration of nitrogen oxides does not affect body weight.

Glucose level in the blood of the animals exposed to nitrogen oxides increased if compared with controls. These differences were statistically significant. At the same time an increased level of lactic acid in the blood was observed (Table 1). A decrease of seromuroid level expressed in mg % of tyrosine was found both in blood serum as well as in the animals exposed to nitrogen oxides.

Table 1
The level of glucose and lactic acid in the blood of control and exposed guinea pigs expressed in mg %

	Control animals $\bar{x} \pm S. D.$	Animals exposed to nitrogen oxides $\bar{x} \pm S. D.$	Statistical significance P
Glucose	78.2 \pm 5.7	87.4 \pm 4.3	< 0.01
Lactic acid	50.3 \pm 4.9	85.2 \pm 7.3	< 0.001

The binding of hexosamines by glycoprotein proteins showed a similar pattern. Hexoses and sialic acids bindings by glycoprotein proteins were increased in the serum, while in the liver the hexose content was increased and that of hexosamines and sialic acids was decreased (Table 2).

The activity of aldolase, lactic dehydrogenase, isocitrate dehydrogenase and phosphohexoisomerase increased both in blood serum as well as in tissues. On the other hand the activity of sorbitol dehydrogenase increased in blood serum and decreased in tissues (Table 3). Numerous urine analyses showed a decrease of pH 7.50 on the average in the animals exposed to nitrogen oxides. In the control group, the average pH was 7.90. No sugar was found in urine. A marked increase of daily elimination with urine of mucopolysaccharides and 3 methoxy — hydroxyamygdalic acid (metabolite of catecholamines) after exposure to nitrogen oxides was very characteristic (Table 4).

*Histochemical investigations**Liver*

Succinic dehydrogenase (SD). A positive fine-grained diffuse reaction was observed in all the liver lobules in the control group (Fig. 1). A

Table 2

The behaviour of seromuroid, hexoses, hexosamines and sialic acids binding by glycoproteid proteins in blood serum (expressed in mg %) and in the liver homogenates (expressed in mg per 100 grams of fresh tissue) in controls and in animals exposed to nitrogen oxides.

		Controls $\bar{x}_1 \pm S. D.$	Animals exposed to nitrogen oxides $\bar{x}_2 \pm S. D.$	Statistical significance P
Seromuroid	serum	6.7 \pm 1.2	4.8 \pm 1.06	< 0.001
	liver	458.6 \pm 42.3	387.2 \pm 34.6	< 0.001
Hexoses binding by glycoproteid proteins	serum	148.6 \pm 9.6	172.6 \pm 16.4	< 0.01
	liver	428.4 \pm 28.0	537.0 \pm 32.0	< 0.001
Hexosamines binding by glycoproteid proteins	serum	118.1 \pm 8.4	97.5 \pm 6.9	< 0.01
	liver	586.0 \pm 38.7	511.0 \pm 34.0	< 0.01
Sialic acid binding by glycoproteid proteins	serum	153.9 \pm 13.1	204.5 \pm 21.2	< 0.001
	liver	204.0 \pm 24.1	151.7 \pm 29.2	< 0.001

granular diffuse product of enzymatic reaction was localized in the cytoplasm of adenocytes, and cellular nuclei were devoid of a positive reaction.

After exposure to nitrogen oxides a slight general decrease of the reaction intensity for S. D. was noticed (Fig. 2).

NADH₂ — tetrazole reductase. A positive fine-grained reaction was noticed in all the liver lobules in the control group. Enzymatic reaction around the veins of the peripheral part of the lobule was stronger than in intermediate and medial zones (Fig. 3). The enzymatic reaction was localized evenly in the cell cytoplasm. After exposure to nitrogen oxides a decrease of the reaction intensity was observed, mainly in the lobule circumference (Fig. 4).

Brain

Succinic dehydrogenase (S. D.) In the control group a positive enzymatic reaction was found both in the nerve cells and in neuroglia. The strongest reaction was observed in the inner-pyramidal layer and in the outer and inner granular layers. In these cells a positive fine-grained-diffuse reaction was localized in the neuroplasm, particularly in the pericaryon. (Fig. 5). After exposure of animals to nitrogen oxides no essential changes were found in the character of localization and intensity of the reaction compared with controls (Fig. 6).

Table 3

The activity of some enzymes in blood serum and in the liver and brain homogenates (per 1 g fresh tissue) expressed in units of a given method

		Control animals $\bar{x}_1 \pm \text{S. D.}$	Animals exposed to nitrogen oxides $\bar{x}_2 \pm \text{S. D.}$	Statistical significance P
Aldolase	serum	109.3 \pm 30.25	178.2 \pm 33.3	< 0.001
	liver	7556.5 \pm 1908.1	10179.7 \pm 1373.7	< 0.01
	brain	6718.7 \pm 1405.2	10980.3 \pm 1464.0	< 0.001
L.D.H.	serum	498.0 \pm 156.2	745.6 \pm 154.5	< 0.001
	liver	18646.0 \pm 4143.6	22745.0 \pm 3976.0	< 0.001
	brain	21600.0 \pm 3579.0	24273.0 \pm 5683.0	< 0.05
P.H.I.	serum	12.3 \pm 1.2	14.6 \pm 1.8	< 0.001
	liver	6490.0 \pm 1083.0	10330.0 \pm 1771.0	< 0.001
	brain	5849.0 \pm 262.0	5988.0 \pm 247.0	< 0.05
S.D.H.	serum	1.5 \pm 0.36	1.9 \pm 0.51	< 0.01
	liver	1331.4 \pm 439.0	1266.0 \pm 189.7	insignificant
	brain	489.6 \pm 61.8	370.0 \pm 48.07	< 0.01
I.D.H.	serum	298.4 \pm 44.8	373.9 \pm 39.3	< 0.001
	liver	44102.0 \pm 366.1	5953.0 \pm 738.0	< 0.001
	brain	1924.0 \pm 207.6	2222.0 \pm 337.9	< 0.001

Table 4

pH of urine and urinary elimination of mucopolysaccharides and of 3 methoxy — 4 hydroxy amygdalic acid in controls and in animals exposed to nitrogen oxides

	Control animals $\bar{x}_1 \pm S. D.$	Animals exposed to nitrogen oxides $\bar{x}_2 \pm S. D.$	Statistical significance P
pH	7.90 \pm 0.4	7.50 \pm 0.3	insignificant
Mucopoly-saccharides (in mg/day)	3.58 \pm 0.7	7.99 \pm 1.9	< 0.001
3 methoxy — 4 hydroxy — amygdalic acid (in mg/kg/24 h)	681.86 \pm 51.0	1237.89 \pm 133.1	< 0.005

NADH₂ — tetrazole-reductase. A positive enzymatic reaction was found in all layers of the examined part of the cortex and in the neuroglia. The strongest enzymatic reaction was localized in granular and in internal pyramidal layers. After exposure to nitrogen oxides no differences were found if compared with controls.

DISCUSSION

The investigations performed have demonstrated that a prolonged exposure of animals to a low concentration of nitrogen oxides increases glucose level in the blood. This phenomenon may be due to an increased secretion of catecholamines under the influence of nitrogen oxides. The increased daily elimination of a metabolite of catecholamines with urine was observed. Adrenaline and to a lesser degree norepinephrine enhance glucose level in the blood by stimulating glycogenolysis in the liver and muscles and they decrease the absorption of glucose by peripheral tissues (18). Adrenaline accelerates glucose synthesis from glycogen in the liver by activating phosphorylase. In the muscles adrenaline reduces the absorption of glucose from the blood, and on the other hand increases both anaerobic glycolysis and elimination of lactates into the blood and liver. The increase of lactic acid level in the blood was found in our investigations after exposure to nitrogen oxides. The investigations performed showed disturbances in the activity of several enzymes which take part in the glycolytic metabolism. The activity of aldolase, lactic dehydrogenase, phosphohexoisomerase and isocitrate dehydrogenase increased after exposure to nitrogen oxides.



Fig. 1.

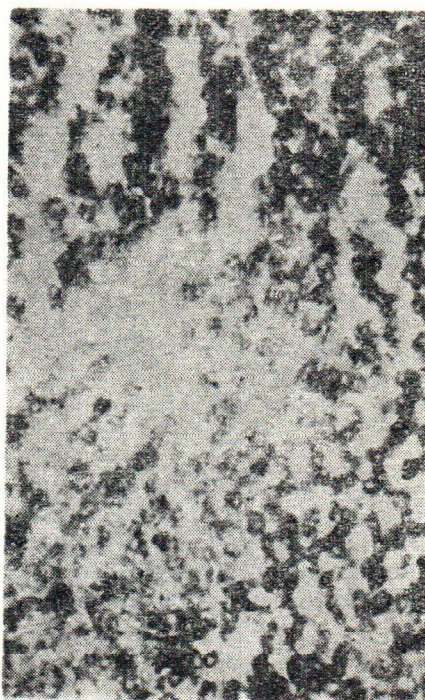


Fig. 2.

Fig. 1. *The liver of a guinea pig from the control group. Positive and fine-grained diffuse reaction for succinic dehydrogenase throughout the lobule (magn. 100 x)*

Fig. 2. *The liver of a guinea pig from the group exposed to nitrogen oxides. The reaction for succinic dehydrogenase shows a slow general decrease of intensity (magn. 100 x)*

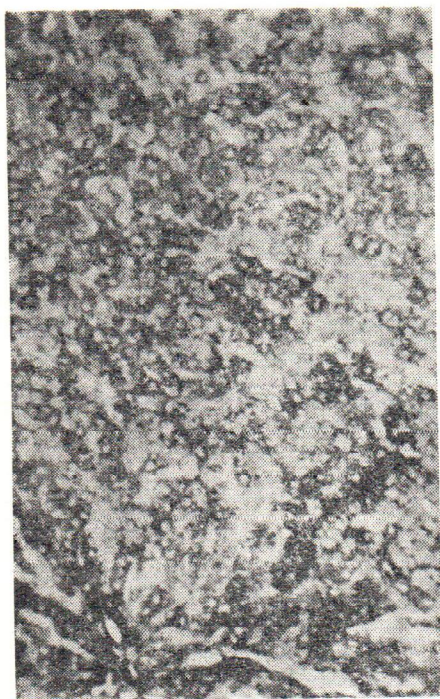


Fig. 3.



Fig. 4.

Fig. 3. *The liver of a guinea pig from the control group. The reaction for NADH₂ — tetrazole reductase is stronger in the region of vessels of the lobule periphery, it is weaker in the medial and intermediate parts. (magn. 100 x)*

Fig. 4. *The liver of a guinea pig from the group exposed to nitrogen oxides. A decrease of the intensity in the reaction for NADH₂ — tetrazole reductase in the peripheral part of the lobule (magn. 100 x)*



Fig. 5.



Fig. 6.

- Fig. 5. *The brain of a guinea pig from the control group. The product of the reaction for succinic dehydrogenase is fine-grained diffuse and localized in the neuroplasm of the region of pericaryon (magn. 400 x)*
- Fig. 6. *The brain of a guinea pig from the group exposed to nitrogen oxides. Localisation and intensity of the reaction for succinic dehydrogenase is similar to that in controls (magn. 400 x)*

The mechanism of this phenomenon has not yet been completely explained. So far only the investigations concerning the mechanism of aldolase increase have been performed. Aldolase is an intracellular enzyme which is usually found in the soluble part of the cytoplasm. The increased activity of this enzyme in blood serum could be due to a biomembrane damage causing a release of the tissue enzyme into the blood. The studies performed by *Ramazzotto* and co-workers (19) indicate that the bubbling of nitrogen dioxide directly into the homogenates of the lung tissue increases aldolase activity. An increase in the enzyme activity depended on the concentration of gases and on the time of exposure. These data may indicate a direct effect of nitrogen oxides on the activity of this enzyme. Until now no *in vitro* investigations have been carried out to explain the mechanism of the activity of other glycolytic enzymes. Parallely with the increase in the activity of glycolytic enzymes we observed a decrease in the activity of the enzyme which takes part in the electron transport i. e. in cellular oxidation. A decrease in the activity of succinic dehydrogenase and NADH₂ tetrazole reductase in the liver was observed in our investigation by means of histochemical methods. The investigations performed *in vitro* by *Ramazzotto* and co-workers (20) demonstrated that the bubbling of nitrogen dioxide into the homogenates of the lungs, liver and kidneys decreased the activity of cytochrome oxidase and succinic dehydrogenase. Cytochrome oxidase being the terminal oxidase in cell respiration and succinic dehydrogenase possibly being the controlling enzyme of the citric acid cycle are of the utmost importance for cell respiration. The observed increase in the activity of glycolytic enzymes may be a result of adaptation of the organism to energy deficiency due to a dissociation of oxidative phosphorylation and oxidation cycle under the influence of nitrogen oxides. The investigations performed have shown that chronic exposure of animals to nitrogen oxides disturbs glycoprotein components. A characteristic feature is a decrease in the seromucoid level, as well as a decrease of hexosamine in blood serum with a simultaneous increase in the binding of hexoses and sialic acids by proteins. The disturbances demonstrated may point to the changes in the liver function produced by nitrogen oxides. An interesting observation was increased elimination of mucopolysaccharides after exposure to nitrogen oxides. The increased secretion of these compounds with urine could be indicative of the impairment of connective tissue, first of all the basement substances. The results of the investigation indicate that nitrogen oxides affect some parameters of carbohydrate metabolism.

CONCLUSIONS

1. Exposure to nitrogen oxides leads to a decreased activity of the enzymes of oxygen transport and simultaneously to an increased activity of the enzymes of glycolytic metabolism.

2. Nitrogen oxides increase the secretion of catecholamines and in this way they may increase glycolysis and glucose level in the blood.
3. Exposure to nitrogen oxides disturbs the metabolism of glycoproteins and mucopolysaccharides.

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Sažetak

UCINAK DUŠIKOVIH OKSIDA NA METABOLIZAM UGLJIKOHIDRATA

Autori su istraživali utjecaj dušikovih oksida na metabolizam ugljikohidrata u zamorčadi. Životinje su eksponirali dušikovu oksidu koncentracije 1 mg/m³ po 8 sati dnevno tijekom 122 dana. Nakon tog vremena mjerili su u krvi tih i kontrolnih životinja 10 različitih parametara važnih u metabolizmu ugljikohidrata. U istih su životinja određivali u mokraći glukozu, mukopolisaharide i metabolite kateholamina. Za nekoliko enzima kojima su aktivnost mjerili u serumu, aktivnost su mjerili i u homogenatima jetre ili mozga. Nekim enzimima u jetri i mozgu aktivnost su mjerili i histokemijskim metodama.

Na temelju dobivenih rezultata autori zaključuju da ekspozicija dušikovim oksidima dovodi do povećanja aktivnosti enzima angažiranih u glikolitičkim procesima a da smanjuje aktivnost enzima angažiranih u prijenosu kisika. Dušikovi oksidi povećavaju sekreciju kateholamina pa i na taj način povećavaju razinu glukoze i ubrzavaju glikolitičke procese. Ekspozicija dušikovim oksidima također remeti i metabolizam glukoproteina i mukopolisaharida.

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Primljeno 2. XI 1976.