Cerebral Oxidized and Reduced Nicotinamide–Adenine Dinucleotide Phosphate and Glucose 6-Phosphate Dehydrogenase in Mice during Exposure to High Oxygen Pressure

By DAROL E. DODD and MORRIS D. FAIMAN

Department of Pharmacology and Toxicology, University of Kansas, Lawrence, KS 66045, U.S.A.

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NADP⁺, NADPH and glucose 6-phosphate dehydrogenase were determined in the cerebral cortex of mice exposed to high O₂ pressure for 0, 8 and 16 min. These time intervals corresponded to 0, 50 and 100% of the CT₅₀ (the time taken for 50% of the mice to convulse). Cerebral NADP⁺, NADPH and glucose 6-phosphate dehydrogenase also were determined in O₂-exposed mice exhibiting hyperactivity, convulsions, and in mice killed 10 s after convulsions. Similar increases in cortical NADP⁺ and decreases in NADPH were found in mice exposed to 610 kPa (6 atm.) of 100% O₂ for 0, 50 and 100% of the CT₅₀, during hyperactivity, onset of seizure and 10 s after convulsions. The NADP⁺/NADPH ratio increased approx. 25% at 0% of the CT₅₀, and remained at this increased value at all O₂-exposure periods including the hyperactive state, onset of seizure and 10 s after convulsions. Identical changes in cerebral NADP⁺, NADPH and the NADP⁺/NADPH ratio were found in mice exposed for 16 min to 100% O₂ at 100, 350 or 610 kPa. No change in cerebral glucose 6-phosphate dehydrogenase was found in mice exposed to 610 kPa of 100% O₂ during the various stages of O₂ toxicity. Only in the 10 s post-convulsive group was a statistically significant decrease in glucose 6-phosphate dehydrogenase observed. Disulfiram [bis(diethylthiocarbamoyl) disulphide], an effective O₂-protective agent, did not prevent the O₂-induced increase in cerebral NADP⁺ and the NADP⁺/NADPH ratio, or decrease in NADPH.

Acute exposure of mammals to O₂ at high pressure produces convulsions and other toxic manifestations. Although the deleterious effects of O₂ have been known since the early work of Bert (1878), the basic mechanism(s) by which O₂ produces convulsions remains unresolved. Numerous theories have been proposed in an attempt to explain the fundamental mechanism(s) of O₂-induced convulsions, and these have been reviewed (Haugaard, 1968).

Hotta (1962) has suggested that GSH may play an important role in brain neuronal integrity, possibly by removal of endogenous peroxides (Christophersen, 1966, 1968). Rapid oxidation of nicotinamide nucleotides by peroxides or other oxidants could affect the glutathione redox system, altering membrane permeability to ions or key substrates and so affect neuronal activity and excitability (Pollen & Trachtenberg, 1970). If changes in the oxidation–reduction state of the glutathione system altered membrane permeability, a control point within the cell could be either the amounts of NADP⁺, NADPH or glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity.

Abbreviations used: GSH, reduced glutathione; CT₅₀, time taken for 50% of mice to convulse.

The role of NADP⁺, NADPH and glucose 6-phosphate dehydrogenase activity in oxygen-toxicity mechanisms has been considered previously. However, these studies have focused primarily on the lung (Willis & Kratzing, 1972; Tierney *et al.*, 1973), liver (Willis & Kratzing, 1972; Chance *et al.*, 1966) and kidney (Chance *et al.*, 1966). Except for one study on the effect of hyperbaric O₂ on cortical NADP⁺ and NADPH (Chance *et al.*, 1965), investigations of the relationship between brain nucleotides and glucose 6-phosphate dehydrogenase, and the susceptibility of animals to O₂-induced convulsions, are non-existent.

In the present study it was hypothesized that hyperbaric O₂ caused NADPH to become oxidized and that this increased oxidation correlated with the susceptibility of mice to O₂-induced convulsions. Experiments were carried out to investigate the effect of hyperbaric O₂ on cortical NADP⁺, NADPH and glucose 6-phosphate dehydrogenase in mice during various stages of central O₂ toxicity. Disulfiram, an effective protectant of O₂ toxicity (Faiman *et al.*, 1971), was used as a pharmacological tool to assess the importance of changes in these nucleotides in the genesis of O₂-induced convulsions.

Experimental

Male Swiss Webster albino mice (HA/ICR) (A.R.S. Sprague-Dawley, Madison, WI, U.S.A.), weighing 25–38 g, were used in all experiments. O₂ exposures were carried out in a hyperbaric chamber in which the mice could be killed inside the chamber without the necessity of decompression (Nolan & Faiman, 1974).

The hyperbaric chamber was constructed from a Plexiglas cylinder, having a capacity of 25 litres. The chamber floor was fitted with a platform containing a trap door opening over a container of liquid N₂. The trap door could be opened at any time by activating an external mechanism. The trap door was opened while the mice were still under O₂ pressure, and the animals fell directly into the liquid N₂. The temperature inside the chamber was maintained at 25±1°C.

As cerebral cortex freezes rapidly in mice of the size used in these studies (Ferrendelli *et al.*, 1972), unshaven mice were used.

In all studies the chamber was flushed with 100% O₂ for 3 min at a flow rate of 12 litres/min to ensure removal of all air. After flushing was completed, the O₂ in the chamber containing the animals was raised slowly to the desired pressure over a 10 min period. Throughout each exposure to O₂, O₂ was passed through the chamber at a rate of 4 litres/min to prevent the accumulation of CO₂, N₂ and water vapour. Exposure of animals was carried out only in the morning to minimize any effects of circadian rhythms.

In studies in which disulfiram [bis(diethylthiocarbamoyl) disulphide] was used, it was administered to mice in a dose of 200 mg/kg intraperitoneally 1 h before their exposure to O₂. Mice not treated with disulfiram were given an equivalent volume (10 ml/kg) of vehicle (polysorbate 80/1% methylcellulose).

Preconvulsive studies

In these studies, cerebral-cortex samples were removed from mice that had been exposed to hyperbaric O₂, but which had not exhibited convulsions or other manifestations of O₂ toxicity. Two mice were placed in the hyperbaric chamber, the chamber was flushed with 100% O₂ and then pressurized to 610 kPa with 100% O₂. As soon as the pressure within the chamber reached 610 kPa, the mice were killed by immersion in liquid N₂. This was referred to as the 'zero' time. Two more mice were placed in the hyperbaric chamber, the same flushing and pressurization procedures repeated, and the chamber was pressurized to 610 kPa. These animals were exposed to O₂ for 2 min, and at the end of this time the animals were killed by immersion in liquid N₂. The same pressurization procedures were repeated so that mice, six in each group, were exposed for 0,

8 and 16 min. These time intervals corresponded to 0, 50 and 100% of the CT₅₀ (the time taken for 50% of the animals to convulse at 610 kPa). Mice exhibiting convulsions or other signs of O₂ toxicity during treatment were discarded and not used in the various CT₅₀ groups. The CT₅₀ was determined before initiation of the studies. Mice were placed inside the chamber, pressurized to 610 kPa with 100% O₂ and the time taken for convulsions to occur was noted. The CT₅₀ value±s.e.m. determined by the method of Oldfield *et al.* (1965) was 16 min.

For studies in which mice were exposed to either 100 or 350 kPa of 100% O₂, similar flushing and pressurization methods were used. In these studies, however, mice were killed after 16 min of exposure to 100% O₂ at either 100 or 350 kPa.

Studies of convulsions and hyperactivity

In these studies, three stages of O₂ toxicity were considered. These were a preconvulsive stage in which mice exhibited hyperactivity, a convulsive stage, as determined by the onset of convulsion, and the post-convulsive stage as measured 10 s after onset of seizures. Two mice were placed inside the hyperbaric chamber, the chamber was pressurized with 100% O₂, and the animals were maintained under pressure until they became hyperactive. At this time, the animals were killed by immersion in liquid N₂. Another two animals were placed in the chamber, pressurized to 610 kPa and killed at onset of seizures. These procedures were repeated for the 10 s post-convulsion group. All exposures were repeated until six animals were obtained for each group.

NADP⁺, NADPH and glucose 6-phosphate dehydrogenase analyses

NADP⁺ and NADPH were determined in cerebral cortex by enzymic cycling methods (Burch *et al.*, 1967; Lowry & Passonneau, 1972). Frozen cerebral samples were dissected and weighed at -20°C. Each nucleotide was assayed in separate tissue samples from the same animal in order that a direct alkali or acidic homogenization could be performed without fear of destruction of one form of cofactor over the other. To prevent oxidation of NADPH, the NADPH samples were homogenized in 0.02 M-NaOH/0.5 mM-cysteine at -10°C and heated for 10 min at 60°C to destroy NADP⁺. The NADP⁺ samples were homogenized in 0.02 M-H₂SO₄/25 mM-ascorbic acid at -10°C and heated for 30 min at 60°C to destroy NADPH. All samples were homogenized by dispersion with a glass rod in small polyethylene tubes. Both NADP⁺ and NADPH standards and blanks underwent the same procedure as tissue samples. To check the overall extraction procedures,

recoveries of NADP⁺ or NADPH added to samples of cerebral tissue were carried out. Recovery for NADP⁺ and NADPH was complete, demonstrating that either NADP⁺ or NADPH could be recovered without serious loss or interconversion. The results for nicotinamide nucleotides were expressed as $\mu\text{mol/kg}$ of frozen cerebrum. Changes in the oxidation-reduction state of the nucleotides during homogenization of frozen cerebral sections were minimized in the present study by determining NADP⁺ and NADPH on separate 10mg samples from the same animal. NADP⁺/acid homogenates were prepared with ascorbic acid to protect against NADPH oxidation by haemoglobin in acid solution (Burch *et al.*, 1967). Changes in the redox state of NADPH and NADP⁺ *in vivo*, due to slow freezing procedures, were minimized by selecting samples from the cerebral cortex, an area of the brain that freezes the fastest (Ferrendelli *et al.*, 1972).

Frozen cerebral-cortex samples for glucose 6-phosphate dehydrogenase analysis (Löhr & Waller, 1974) were homogenized at 4°C in 0.9% NaCl/0.66mM-EDTA. Homogenates were centrifuged at 17600g for 18min, and the supernatants analysed for glucose 6-phosphate dehydrogenase. Soluble protein was determined by the micro biuret method and glucose 6-phosphate dehydrogenase expressed as units of activity/mg of soluble protein, where 1 unit of enzyme activity is the amount of enzyme in 1ml that at 25°C in a 3ml assay mixture changes the A₃₄₀ of NADPH by 0.001 in 1min.

Experimental design and statistical evaluation

Experiments with hyperbaric O₂ only were designed as a one-way analysis of variance. Groups sharing the same treatment were pooled only if an analysis of variance test showed no significant differences between the groups pooled and if variances between groups were shown to be statistically homogeneous. The disulfiram study was designed as a two-way analysis of variance (Sokal & Rohlf, 1969). An *a posteriori* Duncan multiple-range test (Bliss, 1967) was used when a significant *F* value was obtained from statistical treatment of groups studied. Assumptions for performing an analysis of variance were met before statistical testing.

Results

The effect of hyperbaric O₂ on cerebral NADP⁺, NADPH and the NADP⁺/NADPH ratio, and glucose 6-phosphate dehydrogenase activity at various stages of O₂ toxicity, is shown in Figs. 1–3. The NADP⁺/NADPH ratio began to increase soon after the mice were exposed to the high pressure of O₂. For example, by the end of the 3min flush period, the NADP⁺/NADPH ratio was increased by approx.

8%, increasing to 25% by the time the chamber pressure reached 610kPa (0% of the CT₅₀). This increase remained constant during further exposure to O₂ for 50 and 100% of the CT₅₀ (Fig. 1). No further increase in the ratio occurred during the other stages of O₂ toxicity such as the hyperactivity state, the onset of convulsions and the post-convulsive state (Fig. 2). The increased NADP⁺/NADPH ratio reflects the increase in NADP⁺ induced by hyperbaric O₂ and decrease in NADPH. Of interest was the finding that NADP⁺ reached its maximum increase by the time the O₂ pressure reached 610kPa (0% of CT₅₀), and NADPH decreased gradually reaching its maximum decrease at 100% of the CT₅₀. Even at the end of the 3min flush period, there was an approx. 7% increase in NADP⁺, although no change in NADPH was yet apparent. When mice were exposed to 100% O₂ at 100, 350 or 610kPa for 16min, the percentage change in the nucleotides was similar at all three O₂ pressures investigated (Fig. 3).

Disulfiram did not prevent the increase in cortical NADP⁺ and NADP⁺/NADPH ratio observed in mice exposed to hyperbaric O₂ (Table 1). The decrease in NADPH induced by hyperbaric O₂ also was not blocked by disulfiram.

Cerebral glucose 6-phosphate dehydrogenase activity in mice exposed to 610kPa of 100% O₂ increased during all stages of O₂ toxicity, although the increase was not statistically significant. In the

Fig. 1. Cerebral NADP⁺, NADPH and the NADP⁺/NADPH ratio in mice exposed to 100% O₂ at 610kPa. Mice were exposed to 100% O₂ at 610kPa and killed after 0 (0% CT₅₀), 8 (50% CT₅₀) or 16 (100% CT₅₀) min, and cerebral NADP⁺ (●), NADPH (■) and the NADP⁺/NADPH ratio (○) were determined. Each point represents the mean $\pm$ S.E.M. Numbers of mice at each point are given in parentheses. Significance of results: statistically significant at **P* < 0.05 and ***P* < 0.01 when compared with controls.

Fig. 2. Effect of hyperactivity, convulsions and the post-convulsive state on cerebral NADP^+ , NADPH , the $\text{NADP}^+/\text{NADPH}$ ratio and glucose 6-phosphate dehydrogenase activity

Mice were exposed to 100% O_2 at 610 kPa and killed at various stages of O_2 toxicity. Groups included mice killed during hyperactivity (▨), at the onset of convulsions (■), 10s after convulsions (▤), and mice not exposed to hyperbaric O_2 (controls) (□). Each bar represents the mean of six mice $\pm$ S.E.M., except for the control group, which is the mean value of 18 mice. Significance of results: statistically significant at $*P < 0.05$ when compared with controls.

Fig. 3. Effect of O_2 pressure on cerebral NADP^+ , NADPH , the $\text{NADP}^+/\text{NADPH}$ ratio and glucose 6-phosphate dehydrogenase activity

Mice were killed after exposure to 100 kPa, 350 kPa and 610 kPa of 100% O_2 for 16 min and NADP^+ (▨), NADPH (■), the $\text{NADP}^+/\text{NADPH}$ ratio (▤) and glucose 6-phosphate dehydrogenase activity (□) were determined. Each bar represents the mean of six mice, except for the control group, which is the mean value for 12 mice. Mice exposed to 610 kPa of 100% O_2 were compared with the following control values: NADP^+ ($10.8 \pm 0.2 \mu\text{mol/kg}$); NADPH ($24.6 \pm 0.5 \mu\text{mol/kg}$); $\text{NADP}^+/\text{NADPH}$ (0.44 ± 0.01); glucose 6-phosphate dehydrogenase (55.3 ± 2.5 units/mg of protein). Mice exposed to 100 kPa and 350 kPa of 100% O_2 were compared with the following control values: NADP^+ ($10.9 \pm 0.6 \mu\text{mol/kg}$), NADPH ($25.7 \pm 0.07 \mu\text{mol/kg}$), $\text{NADP}^+/\text{NADPH}$ (0.42 ± 0.02) and glucose 6-phosphate dehydrogenase (61.0 ± 4.9 units/mg of protein). Significance of results: statistically significant at $**P < 0.01$ when compared with controls.

post-convulsive state a statistically significant decrease ($P < 0.05$) occurred (Figs. 2 and 4).

Discussion

Hyperbaric O_2 increased both cortical NADP^+ and the $\text{NADP}^+/\text{NADPH}$ ratio, and decreased NADPH at all stages of O_2 exposure (Figs. 1 and 2). Changes in these nucleotides appear to occur quite rapidly as only 13 min had elapsed from the time the mice were placed in the hyperbaric chamber until 610 kPa was reached. The increased $\text{NADP}^+/\text{NADPH}$ ratios were generally associated with significant decreases in NADPH . It thus appears that oxidation of NADPH occurs as soon as animals are exposed to increased O_2 pressures, with the $\text{NADP}^+/\text{NADPH}$ ratio reaching a constant value and remaining at this value. Chance *et al.* (1966) found the cortical $\text{NADP}^+/\text{NADPH}$ ratio from rats exposed to 810 kPa of 100% O_2 for 16 min to be increased by approx. 80%, which is consistent with our data. Of considerable interest was the observation that changes in NADP^+ , NADPH and the $\text{NADP}^+/\text{NADPH}$ ratio do not appear to be related to O_2 pressure since similar changes were found when mice were exposed to 100, 350 or 610 kPa of 100% O_2 (Fig. 3).

Mayevsky *et al.* (1974), using surface fluorescence techniques, carried out studies *in situ* with rats exposed to 610 kPa of 100% O_2 . They found an oxidized nicotinamide nucleotide stage that occurred before seizures, an oxidized stage concurrent with convulsions, and an oxidation wave after seizures. Further, the oxidation-reduction state of the nicotinamide

Table 1. Effect of disulfiram and disulfiram plus O₂ on cerebral NADP⁺, NADPH and glucose 6-phosphate dehydrogenase activity

Mice were exposed to 100% O₂ at 610 kPa for 16 min. Disulfiram (200 mg/kg) was injected intraperitoneally 1 h before O₂ exposure. Non-O₂-exposed and disulfiram-treated mice also were killed 1 h after drug administration. Values are mean $\pm$ S.E.M. Significance of results: statistically significant at * P < 0.05 and ** P < 0.01 when compared with controls.

Treatment	No.	NADP ⁺ (μ mol/kg)	NADPH (μ mol/kg)	NADP ⁺ /NADPH ratio	Glucose 6-phosphate dehydrogenase (units/mg of protein)
Control	6	13.2 $\pm$ 0.7	26.4 $\pm$ 0.5	0.50 $\pm$ 0.03	58.2 $\pm$ 2.2
O ₂	6	15.7 $\pm$ 0.6	24.0 $\pm$ 1.1	0.66 $\pm$ 0.03*	57.7 $\pm$ 3.3
Disulfiram	6	14.0 $\pm$ 1.1	25.2 $\pm$ 1.5	0.56 $\pm$ 0.05	49.7 $\pm$ 3.2
Disulfiram + O ₂	6	16.4 $\pm$ 1.1*	24.2 $\pm$ 2.2	0.69 $\pm$ 0.05**	53.9 $\pm$ 4.8

Fig. 4. Cerebral glucose 6-phosphate dehydrogenase activity in mice exposed to 100% O₂ at 610 kPa

Mice were exposed to 100% O₂ at 610 kPa and killed after 0 (0% CT₅₀), 8 (50% CT₅₀) or 16 (100% CT₅₀) min. Each point represents the mean $\pm$ S.E.M. for the numbers of mice at each point given in parentheses.

nucleotides correlated with the electrical activity in the brains of rats exposed to hyperbaric O₂. Unfortunately, surface-fluorescence techniques do not distinguish between NADPH and NADH. Recent findings indicate that essentially all of the labile surface fluorescence originates from mitochondrial NADH (Jöbsis *et al.*, 1971; O'Connor *et al.*, 1972; O'Connor, 1977). Since the contribution of cytoplasmic NADPH in surface fluorimetry has not been completely assessed, our findings with NADP⁺ and NADPH are comparable with respect to oxidation-reduction trends only.

Although hyperbaric O₂ increased cerebral NADP⁺ and the NADP⁺/NADPH ratio, with a concomitant decrease in NADPH, these changes do not appear to correlate with O₂-induced convulsions. This is suggested by the finding that similar increases in cerebral NADP⁺ and decreases in NADPH were found in mice exposed to 0, 50 or 100% of the CT₅₀, hyper-

activity, seizure onset, and 10s post-ictally. Also mice exposed to 100, 350 and 610 kPa of 100% O₂ for 16 min all exhibited similar changes in NADP⁺ and NADPH (Fig. 3). Yet the time taken for 50% of the mice to convulse when exposed to O₂ at 100, 350 and 610 kPa was 960 min (Faiman & Heble, 1966), 102 min (D. E. Dodd & M. D. Faiman, unpublished results) and 16 min respectively. Thus similar changes in nucleotides were found in mice killed after exposure to O₂ at 100, 350 or 610 kPa even though at 100 and 350 kPa the mice were killed considerably sooner than their expected convulsion time. Finally, disulfiram, which effectively protects against O₂-induced convulsions in both mice (Faiman *et al.*, 1971) and dogs (Faiman *et al.*, 1974), did not prevent the increase in cortical NADP⁺ and NADP⁺/NADPH ratio or decrease in NADPH induced by hyperbaric O₂ (Table 1).

The reason for the increase in the NADP⁺/NADPH towards ratio oxidation is uncertain. Torbati *et al.* (1976) found cortical *p*O₂ in rats to be increased as the O₂ pressure in the hyperbaric chamber increased, with the *p*O₂ reaching a maximum during the pressurization stage of exposure to hyperbaric O₂. The onset of the first electrical discharge, which reflected central O₂ toxicity, did not alter cortical *p*O₂. Similar findings have been observed by others (Ogilvie *et al.*, 1976). Our results seem to correlate well with these O₂-availability observations, in that the NADP⁺/NADPH ratio increased immediately, reached a maximum oxidation at 0% of the CT₅₀ (610 kPa) and remained at a constant value throughout the various stages of central O₂ toxicity including post-seizures.

Cerebral glucose 6-phosphate dehydrogenase activity remained normal in non-convulsed mice exposed to O₂ at 100, 350 and 610 kPa (Figs. 2, 3 and 4). This observation does not agree with studies on the role of glucose 6-phosphate dehydrogenase activity in pulmonary O₂ toxicity as others have found O₂ to increase glucose 6-phosphate dehydrogenase activity (Tierney *et al.*, 1973, 1975; Kimball *et al.*,

1976; Bassett & Fisher, 1976). This may be explained by differences in times to which the animals were subjected to O_2 . In our studies mice were exposed to hyperbaric O_2 for short periods, that is, less than 30 min. In the studies on the role of glucose 6-phosphate dehydrogenase activity in pulmonary O_2 toxicity, animals were exposed to 85–90% O_2 at 100 kPa for 7–14 days (Tierney *et al.*, 1973, 1975; Kimball *et al.*, 1976; Bassett & Fisher, 1976) before an increased glucose 6-phosphate dehydrogenase activity was observed. In perfused rat lung, Nishiki *et al.* (1976) found that hyperbaric O_2 produced a marked release of oxidized glutathione, and these investigators suggested that the glutathione oxidation–reduction enzyme system plays an important role in detoxifying lipid peroxides produced in the lung cell. The hexose monophosphate shunt in lungs of rats exposed to chronic hyperoxia may play a vital role in providing NADPH-reducing power for tissue repair and proliferation. In this way NADPH may play a critical role in maintaining glutathione in a reduced state in the lung cell. However, this does not appear to be the case for the brain, and it appears that different mechanisms are operative in central nervous system and pulmonary O_2 toxicity.

Disulfiram produced an approx. 15% decrease in glucose 6-phosphate dehydrogenase activity (Table 1), although the decrease was not statistically significant. This is not surprising since disulfiram has been shown to be an effective inhibitor of other dehydrogenase enzymes *in vitro* (Hassinen, 1967) and *in vivo* (Deitrich & Erwin, 1971). Further, disulfiram has been reported to inhibit glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) in non-mammalian studies (Chefurka, 1957).

Our results show a marked decrease in cerebral glucose 6-phosphate dehydrogenase activity in mice after convulsions induced by hyperbaric O_2 . The reason for this is not apparent at this time. King *et al.* (1973) suggested that glucose 6-phosphate dehydrogenase may be inhibited during maximal electroshock-induced convulsions in mice. In studies *in vivo*, Banister *et al.* (1973) found decreased activity of brain lactate dehydrogenase, cytochrome oxidase and NADH dehydrogenase in rats convulsed by exposure to hyperbaric O_2 . These observations suggest that enzyme inhibition by such convulsions may lead to changes in the nicotinamide nucleotides redox state.

The primary mechanism for the observed decrease in NADPH and increase in cortical $NADP^+$ and the $NADP^+/NADPH$ ratio found in our studies with hyperbaric O_2 remains unresolved. Further, the importance of the respective decreases and increases in the initiation of O_2 -induced convulsions is uncertain. Although it appears that the $NADP^+/NADPH$ system is not directly related to the onset of O_2 -induced convulsions, changes in these nucleotides

may be contributory to increased excitability and seizure onset.

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