The Effects of Halothane on Metabolism and Calcium Uptake in Mitochondria of the Rat Liver and Brain

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The actions of halothane on mitochondrial respiration and on the uptake of calcium by brain mitochondria were studied in vitro. Halothane markedly inhibited ADP-stimulated respiration of both liver and brain mitochondria. The sensitivity to inhibition by halothane was greater with glutamate than with succinate as substrate. Halothane is an inhibitor of oxidative phosphorylation and not an uncoupling agent, except in some circumstances at very high concentrations. In liver mitochondria halothane effectively inhibited the increases in rates of respiration produced by calcium and phosphate when glutamate was the respiratory substrate. This effect was not seen with mitochondria respiring in the presence of succinate. Calcium uptake by brain mitochondria was inhibited by halothane when the mitochondria were incubated in the presence of ATP, ATP plus glutamate and phosphate, and ATP plus succinate and phosphate. With succinate as the substrate, the inhibitory action of halothane was preceded in time by a temporary stimulation of calcium uptake. Much additional experimentation is needed to determine whether halothane influences calcium movements in the brain in vivo and whether such actions of a general anesthetic agent play a role in the production of the anesthetic state in the intact organism. (Key words: Brain; Halothane; Liver; Mitochondria; Theories of anesthesia.)

At present, the cellular basis for the phenomenon of anesthesia is unknown. Some investigations have been concerned with the possibility that anesthetic agents may interfere with electron transport, energy production, or other processes catalyzed by mitochondria. Chance and Hollunger demonstrated that Amytal inhibits electron transport in mitochondria from different tissues and species at a flavoprotein site involved in the oxidation of NADH. More recently, several investigators have shown that halothane inhibits respiration by liver mitochondria at the site of the NAD− flavoprotein−coenzyme Q reactions in the electron transport chain. Other anesthetic agents, e.g., diethyl ether and methoxyflurane, have been observed to depress respiration of liver mitochondria with either succinate or glutamate as substrate.

Mitochondrial function in the cell involves not only the formation of energy in the form of ATP but also the uptake and release of inorganic ions. In particular, the sequestration of calcium ions by mitochondria may play a role in the regulation of cellular reactions in vivo. Chance, Mela and Harris have shown recently that local anesthetic agents can influence the uptake of calcium by mitochondria and in this way these substances may alter cell function.

The present investigation was undertaken to determine whether halothane affects the metabolism of brain mitochondria in a manner similar to that observed with mitochondria from liver and also to study whether halothane influences the ability of brain mitochondria to take up calcium in vitro.

Materials and Methods

Mitochondria were prepared from liver and brain obtained from male Wistar rats fed ab lib. The liver mitochondria were isolated in 0.25 M sucrose by the method of Hogeboom as modified by Myers and Slater. Brain mitochondria are more difficult to separate from other tissue constituents. The method used was that reported by Ozawa et al., which employs a medium consisting of 0.18 M mannitol. We have modified this method, and in

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our hands \textsuperscript{12,14} the preparations finally obtained contain mitochondria as the major fraction (as indicated by electron microscopy and respiratory control values). Almost no myelin or microsomes are present, but there is a definite admixture of synaptosomes from nerve cell endings. These bodies, which contain one or more mitochondria each, sediment at about the same rate in the centrifuge as mitochondria and are therefore extremely difficult to exclude from the mitochondrial preparations.

Protein content of the mitochondrial suspensions was determined with biuret as described by Cleland and Slater.\textsuperscript{12} The protein concentrations in the mitochondrial preparations ranged from 10 to 20 mg/ml for liver mitochondria and from 3.5 to 10 mg/ml for brain mitochondria.

**Preincubation of Mitochondria**

In the studies of the action of halothane, the mitochondria were preincubated in 25-ml Erlenmeyer flasks with center wells. The mitochondrial suspension (0.5 ml) was added to the main compartment of the vessel, which contained 1.5 ml of a buffered solution consisting of 0.040 M HEPES (N-2-hydroxyethyl piperazine N\textsuperscript{1}-2-ethane sulfonic acid) at pH 7.4, 0.010 M MgCl\textsubscript{2}, and 0.105 M KCl, and the flask was shaken in a water bath at 25 C. The anesthetic was added to the center well immediately before preincubation and the flask covered with Mylar.

**Studies of Mitochondrial Respiration**

Samples of the preincubation mixture were removed and placed in the chamber of a Gilson oxygraph with a Clark oxygen electrode. The rate of oxygen uptake was measured at 26 C in the presence of glutamate or succinate as substrate and also after the further addition of inorganic phosphate and ADP. The first determination (state-4 respiration) provides a measure of the rate of oxygen uptake when mitochondrial metabolism is restrained by the absence of phosphate acceptor, while the latter determination (state-3 respiration) measures the rate of oxygen uptake in the presence of excess phosphate and phosphate acceptor. A substance causing uncoupling of oxygen uptake and energy formation increases the rate of state-4 respiration, while an inhibitor of oxidative phosphorylation specifically inhibits state-3 respiration.

Mitochondrial respiration is stimulated not only by ADP in the presence of phosphate but also by the addition of CaCl\textsubscript{2}. In order to determine the effect of halothane on calcium-stimulated respiration, experiments in which oxygen uptake was measured after addition of CaCl\textsubscript{2} and inorganic phosphate were also performed. In all experiments the mitochondrial suspensions were preincubated in the absence of halothane as controls, and experiments were also carried out with mitochondria kept at 0 C before the measurements.

In some experiments the ATP content of the reaction mixture was measured at the end of the incubation. One ml of the suspension was removed from the reaction chamber and added to 3 ml 0.625 N cold perchloric acid (PCA). After neutralization and removal of potassium perchlorate, the ATP was measured by a specific enzymatic method.\textsuperscript{16} In these experiments, the P/O ratio, the high-energy phosphate formed per atom oxygen consumed was calculated.

**Studies of Calcium Uptake**

In these experiments the mitochondria were preincubated in Erlenmeyer flasks with center wells in the absence and presence of different concentrations of halothane. A 0.1-ml amount of the mitochondrial preparation was added to approximately 1.3 ml of medium, which contained, in addition to HEPES buffer, MgCl\textsubscript{2} and KCl, and also ATP, ATP plus glutamate and phosphate, or ATP plus succinate and phosphate. The concentrations of the reaction components are given in the legends to the figures.

After 4 minutes of preincubation at 25 C, CaCl\textsubscript{2} was added to the reaction mixture (final concentration 0.4 mM). The contents of the flasks were filtered through 0.45 \textmu M Millipore filters, 0.5, 1.5, and 3 minutes after the addition of CaCl\textsubscript{2}. The time of filtration was very rapid (5 seconds or less) so that redistribution of calcium would be minimal. The filtrate was diluted 11 times with 0.01 M SrCl\textsubscript{2}-0.1 N HCl solution, and calcium in the filtrate was determined with a 290 B Perkin-Elmer atomic absorption spectrophotometer. The decrease in calcium content of the medium, corresponding
Fig. 1. The effect of halothane on the stimulation of respiration of rat liver mitochondria by calcium, with glutamate as substrate. Mitochondrial suspension, 0.2 ml, was preincubated with 0.6 ml HEPES buffer medium for 5 minutes at 25°C. The halothane concentration during preincubation is indicated. Oxygen uptake was determined at 26°C with 0.6 ml of this suspension after addition of potassium phosphate (1.33 mM), sodium glutamate (6.7 mM) and additional HEPES buffer to give a final volume of 1.48 ml. CaCl₂ or succinate was added at arrows. The final mitochondrial protein concentration was 4.40 mg/ml; HEPES (pH 7.4), 0.032 M; MgCl₂, 0.008 M; KCl, 0.086 M; sucrose, 0.036 M. Rates of oxygen uptake are given next to curves in μatoms O/min/ml solution.

Fig. 2. The effect of halothane on the stimulation of respiration of rat liver mitochondria by calcium, with succinate as substrate. Experimental conditions identical to those described in the legend to figure 1 except that succinate (6.7 mM) was present as substrate and rotenone (1 μg/ml) was added to suppress endogenous respiration. The final mitochondrial protein concentration was 3.3 mg/ml. Experimental results are reported as described in the legend to figure 1.
HALOTHANE EFFECTS ON LIVER AND BRAIN MITOCHONDRIA

SUBSTRATE: Glutamate

0.17 mg/ml HALO
0.86 mg/ml HALO

0.38 mM ADP
0.25 μ moles ATP/ml
0.31 μ moles ATP/ml
0.30 μ moles ATP/ml

SUBSTRATE: Succinate

0.17 mg/ml HALO
0.86 mg/ml HALO

0.38 mM ADP
0.25 μ moles O
0.30 μ moles ATP/ml
0.30 μ moles ATP/ml
0.14 μ moles ATP/ml

Fig. 3. The effect of halothane on respiration of rat brain mitochondria. Glutamate or succinate as substrate. During exposure to halothane 0.3 ml of mitochondrial suspension was incubated with 0.9 ml HEPES buffer medium for 5 minutes at 25 C; 0.8 ml of this suspension was used for measurement of oxygen uptake. HEPES buffer was employed to bring the total volume to 1.3 ml after adding mitochondria and glutamate or succinate (to 7.5 mM). ADP, to 0.38 mM, and potassium phosphate to 1.47 mM, were added at arrows. The final concentration of mitochondrial protein was 0.66 mg/ml; HEPES (pH 7.4), 0.030 M; MgCl₂, 0.008 M; KCl, 0.078 M; mannitol, 0.028 M. Experimental results are reported as described in the legend to figure 1.

to the uptake of calcium by the mitochondria, was plotted as a function of time.

MEASUREMENT OF HALOTHANE CONCENTRATION

The concentration of halothane in the mitochondrial reaction mixtures was determined in some experiments by the method of Butler et al.,¹⁷ using a Varian aerograph gas chromatograph. Incubation mixtures with mitochondria were incubated in Erlenmeyer flasks at 25 C for 5 minutes with various amounts of halothane pipetted into the center well. Samples of the fluid were taken for chromatographic determination of halothane. Results were within ±0.05 mg/ml of the concentrations predicted from the volumes of halothane added. The partition coefficient was such that 1 mg halothane per 1 ml reaction mixture corresponded to 9.5 per cent halothane in the gas phase.

Experimental Results

EFFECTS OF HALOTHANE ON RESPIRATION OF LIVER MITOCHONDRIA

Before studying the effects of halothane on brain mitochondria we carried out a number of experiments with mitochondria isolated from rat liver in order to observe the action of the
anesthetic in this well-studied preparation and to obtain data that could be compared with the results of subsequent experiments with brain mitochondria. We were particularly interested in determining whether halothane caused uncoupling of oxidation and phosphorylation or whether it acted primarily as an inhibitor of oxidative phosphorylation. If the latter were true, halothane should inhibit the increase in respiration seen when either ADP or calcium is added to mitochondria respiring in the presence of substrate and phosphate ions.

In confirmation of the results of others, we found that halothane inhibited the rate of oxygen uptake of liver mitochondria during state-3 respiration in the presence of glutamate or succinate as substrate. However, at any given concentration of anesthetic, the extent of inhibition was greater with glutamate than with succinate as substrate. For example, a concentration of 0.18 mg halothane/ml that caused 50 per cent inhibition of state-3 respiration with glutamate as substrate had no significant effect in the presence of succinate. A concentration of 0.40 mg halothane/ml prevented almost completely the stimulation of respiration produced by ADP and phosphate in the presence of glutamate, but inhibited respiration with succinate by only 30 per cent. Measurements of ATP production showed that this process was inhibited to about the same extent as the oxygen uptake, so that the phosphate esterified per atom of oxygen consumed (the P/O ratio) remained constant. These experiments show conclusively that halothane, when added to liver mitochondria, is an inhibitor of oxidative phosphorylation and not an uncoupling agent. Only in the presence of succinate and at a very high concentration of halothane (0.89 mg/ml) was there an increase in respiration in the absence of ADP and no significant ATP formation, i.e., uncoupling of respiration and phosphorylation. Since the results reported here are mainly confirmatory in nature, we have not included the actual data.

When calcium is added to mitochondria respiring in the presence of substrate there is a small transient increase in oxygen uptake. However, when phosphate is present or added with the calcium, the stimulation of respiration is closely related to the energy-requiring entrance of both calcium and phosphate ions into the mitochondria, and is much larger and more prolonged than when calcium is added alone. The influx of phosphate ions into mitochondria is a step common to oxidative phosphorylation and calcium stimulation of respiration in the presence of phosphate. If halothane inhibited oxidation of glutamate to a greater extent than succinate-supported respiration, this anesthetic would be expected to influence calcium-stimulated respiration to a much greater degree with glutamate than with succinate as substrate. This was found to be the case. The experiments with glutamate are recorded in figure 1.

In the absence of halothane, the addition of CaCl₂ was followed by a marked rise in the rate of oxygen consumption and a later return to a slower rate. When the mitochondria had been preincubated for 5 minutes in the presence of 0.05 mg/ml or 0.46 mg/ml halothane, the addition of calcium no longer increased the rate of respiration, and there was an inhibition of oxygen uptake after about 2 minutes. The addition of succinate at this point led to a marked increase in the rate of respiration. The results were quite different when succinate was present as a substrate (fig. 2).

As in the experiments reported in figure 1, the mitochondrial suspensions were preincubated in the absence of and in the presence of halothane at different concentrations. The only difference was that the substrate present during the measurement of respiration was succinate rather than glutamate. Rotenone, an inhibitor of the oxidation of NAD-linked metabolites, was added to suppress the metabolism of endogenous substrates.

As with glutamate, the addition of CaCl₂ increased the rate of respiration of control mitochondria. The oxygen uptake returned toward normal after about a minute, but the addition of more CaCl₂ again stimulated respiration. With mitochondria pre-exposed to 0.17 mg halothane/ml, the results were essentially similar to those observed with control mitochondria. When the mitochondria had been exposed to 0.43 mg halothane/ml (a concentration of the anesthetic causing complete inhibition of calcium-stimulated respiration with glutamate as substrate), there was, if anything, a greater action of calcium in stimulating respiration. The mitochondria exposed to the
very high concentration of halothane of 0.86 mg/ml were uncoupled, and calcium had no effect on the already elevated rate of oxygen uptake.

In general, the studies with liver mitochondria showed that in the presence of the NAD-linked substrate, glutamate, halothane inhibits both ADP- and calcium-stimulated respiration. With succinate as substrate, halothane was less potent as an inhibitor of ADP-stimulated respiration, and it did not inhibit the increase in respiration seen after addition of calcium and phosphate.

**Effects of Halothane on Respiration of Brain Mitochondria**

The effect of preincubation of brain mitochondria with halothane was studied in experiments similar to those carried out with mitochondria from liver. Representative experiments are recorded in figure 3.

It is evident that with either glutamate or succinate as substrate the action of halothane is that of inhibiting the ADP-stimulated respiration. As with liver mitochondria, the extent of the inhibition, in per cent of the control rate of respiration, was greater with glutamate than with succinate as substrate. The measurement of ATP at the end of the incubation showed that halothane did not abolish the phosphorylation of ADP but diminished the rate of this reaction. In other words, halothane acted as an inhibitor of oxidative phosphorylation and not as an uncoupling agent. In contrast to the results with liver mitochondria, the very high concentration of halothane of 0.86 mg/ml did not produce uncoupling of respiration in the presence of succinate.

**Effect of Halothane on Calcium Uptake by Brain Mitochondria**

The ability of mitochondria to take up and release calcium ions is probably of considerable physiologic importance. The influx of calcium into mitochondria can be supported by energy from respiration or by energy supplied by ATP. In contrast to liver mitochondria, mitochondria from brain take up very little calcium in a medium devoid of ATP. However, when a metabolizable substrate and inorganic phosphate are added to a reaction mixture already containing ATP, there is a further large increase in the rate of calcium uptake. The effect of halothane on mitochondrial calcium transport was therefore studied in the presence of ATP alone, in the presence of ATP, glutamate, and phosphate, and in a medium containing ATP, succinate, and phosphate. In each series of experiments the mitochondrial reaction mixtures were exposed to halothane at 25 C for 4 minutes and CaCl₂ then added. The disappearance of calcium was determined after various periods of further incubation at 25 C. The results are reported in figures 4 to 6.

The results with ATP, glutamate, and phosphate are shown in figure 4. It is evident that there is a dose-dependent inhibition of calcium uptake by halothane. It may be important that the rapid uptake during the first 30 seconds of incubation is particularly sensitive to inhibition by the anesthetic. At the high concentration of 1.7 mg halothane/ml the initial slow uptake of calcium is followed by extrusion of the calcium ions from the mitochondria.

The results with mitochondria incubated in the presence of ATP, succinate, and phosphate are recorded in figure 5. The results were quite different from those in the experiments with glutamate as substrate, in that there was a consistent increase rather than a decrease in the rate of calcium uptake during the first 30 seconds of incubation even at the highest concentration of anesthetic. This was followed by an inhibition of calcium transport. As with glutamate, there was extrusion of the calcium taken up when the highest concentration of halothane (1.7 mg/ml) was used. The difference between the effects of halothane on calcium uptake with glutamate and with succinate as substrate are in agreement with the effects of the anesthetic on respiration. The ADP-stimulated oxygen was much more sensitive to inhibition by halothane with glutamate than with succinate as substrate.

The results obtained when ATP were present alone are shown in figure 6. In these experiments there was a progressive inhibition of calcium uptake as the halothane concentration was increased, indicating that ATP-supported calcium transport, as well as respiration-supported calcium uptake, is inhibited by halothane.
**Fig. 4.** Calcium uptake by brain mitochondria in the presence of glutamate, ATP, and inorganic phosphate. Each point represents the mean of the results from six experiments and is the difference between the mean initial calcium concentration and the mean calcium concentration after a given period of incubation.

The mitochondrial suspension, ATP, glutamate, and inorganic phosphate were preincubated with halothane for 4 minutes at 25°C. CaCl₂ was then added and Millipore filtration carried out 1/₂, 1, ½ or 3 minutes later. Total fluid volume was approximately 1.35 ml. Halothane concentration is in mg/ml. The reaction mixture contained: glutamate, 6.9 mM; ATP, 6.9 mM; inorganic phosphate, 6.8 mM; HEPES buffer, 0.030 M; KCl, 0.080 M; MgCl₂, 0.005 M. The mean final mitochondrial protein concentration was 0.40 mg/ml. The initial calcium concentration in these experiments and in the experiments reported in figures 5 and 6 was about 0.45 mM.

**Fig. 5.** Calcium uptake by brain mitochondria in the presence of succinate, ATP, and inorganic phosphate. The experimental procedure was as described in the legend to figure 4. The composition of the reaction mixtures was as in figure 4 except that succinate rather than glutamate was added as substrate. Results are means from three experiments. The mean mitochondrial protein concentration was 0.45 mg/ml.

**Discussion**

The results of the experiments reported here confirm previous findings that halothane has the ability to inhibit electron transport in liver mitochondria incubated in vivo. The inhibition of respiration is particularly evident when ADP and phosphate are added to the mitochondria. Under these conditions control mitochondria respond with a marked increase in the rate of respiration (state-3 respiration), while mitochondria pre-exposed to halothane are stimulated by ADP and phosphate to a much lesser extent. Halothane, therefore, has the properties of an inhibitor of oxidative phosphorylation, not those of an uncoupling agent. The inhibition of ADP-stimulated respiration
Fig. 6. Calcium uptake by brain mitochondria in the presence of ATP alone. The experimental procedure was as described in the legend to figure 4 except that no substrate or phosphate was added. Results are means from five experiments. The concentration of ATP was 7.35 mM. The mean mitochondrial protein concentration was 0.35 mg/ml.

was greater with glutamate than with succinate as substrate, indicating that NAD-linked reactions are more sensitive to inhibition by halothane than reactions more distant in the electron-transport chain.

The action of halothane on the respiration of brain mitochondria was similar to the effect of this anesthetic on liver mitochondria except that the inhibition of respiration with succinate was probably greater than with liver mitochondria. It was also observed that with brain mitochondria halothane always inhibited oxidative phosphorylation and did not act as an uncoupling agent even at high concentrations.

The ability of brain mitochondria to sequester calcium was shown to be markedly inhibited by prior incubation of the mitochondria in the presence of halothane. This defect in calcium transport by brain mitochondria exposed to halothane was clearly evident when the mitochondria were incubated with ATP alone and when they were incubated with ATP, phosphate, and the metabolites succinate or glutamate. Apparently, both ATP-supported uptake of calcium and respiration-supported uptake of calcium are depressed by halothane. It should not be overlooked, however, that pre-exposure to low concentrations of halothane caused an initial increase in the rate of calcium uptake by brain mitochondria incubated with succinate as the respiratory substrate. The meaning of this phenomenon is not clear, but it was observed consistently.

The concentrations of halothane at which the effects in vitro were obtained are close to or within the range of those that can be expected during anesthesia in the intact organism. Blood halothane concentrations during anesthesia in man have been reported to be about 0.2 mg/ml, and in dogs 0.5 mg/ml has been observed. Wolfson et al. measured the concentrations of halothane in the brains of rats anesthetized with this agent and found that in animals that did not respond to stimuli the brain content of halothane was, on the average, 0.29 mg per gram of tissue. Our experiments show that concentrations of halothane in this range markedly influence mitochondrial respiration and calcium transport.

The effects of interference with mitochondrial function in vitro by an anesthetic agent are difficult to predict. It seems unlikely that inhibition of ATP formation by mitochondria can play a significant role in the production of anesthesia, since the brain ATP concentration is not depressed during the anesthetic state, and a substance such as dinitrophenol, the classic uncoupler of oxidative phosphorylation,
does not produce anesthesia. It is more likely that a decrease in the rate of calcium uptake by brain mitochondria, as produced by halothane, could influence neuronal activity. Such an event, if it occurred in *vivo*, could be expected to have many effects on both metabolism and function of neurons in the central nervous system.

There is much evidence that changes in calcium concentration can alter the functional state of excitable membranes.\textsuperscript{24, 25, 26} During the resting state, calcium ions are associated with the membranes and there is low permeability to cations. Upon calcium dissociation from the membrane, permeability to ions increases. If halothane decreased the ability of neuronal mitochondria in *vivo* to take up calcium, one would expect a shift in the internal calcium stores, with a decrease in mitochondrial calcium and an increase in the cytoplasmic concentrations of free calcium. Since the concentration of calcium in the cytoplasm is very low (of the order of $10^{-7}$ M) a very slight inhibition of mitochondrial calcium uptake could produce relatively large changes in extramitochondrial calcium in the cell.

The results of experiments on the introduction of calcium into the cell are very pertinent to our discussion. Meech and Strumwasser\textsuperscript{27} found an increase in potassium conductance of the membrane when calcium was instilled into the ganglion of *Aplysia*, with resultant hyperpolarization. Tasaki et al.\textsuperscript{28} demonstrated that perfusing the interior of the squid axon with a medium containing calcium ions led to a lowering of the resistance of the axonal membrane and a significant and reversible decrease in the size of the action potential. Results with tissues containing tight junctions show that an increase in the cell content of free calcium ions can depress conductance of impulses from one cell to its adjoining cell. Lowenstein et al.\textsuperscript{29} demonstrated that when calcium was introduced into salivary gland cells by iontophoresis or through leaks produced in the cell-surface membrane, the permeability of junctional membranes to ions decreased by one to three orders so that they approached nonjunctional membranes in their permeability characteristics.

The studies of Krnjevic and Lisiewicz\textsuperscript{30} also provide evidence that an increase in the concentration of cellular calcium diminishes cellular activity. These authors injected CaCl$_2$ into lumbosacral motoneurons of cats and observed a reduction in excitability and a decrease in membrane resistance that were both rapid in onset and quickly reversible. They speculated that the release of calcium from intraneuronal stores such as mitochondria could serve a protective function in the cell by stabilizing the cell membrane, during hypoxia or when cell metabolism is depressed by specific inhibitors of respiration.

There are many possible ways in which an excess of intracellular calcium could alter neuronal function. The action could be primarily on the cell membrane to produce stabilization and decrease excitability. However, it is also possible that an excess of calcium ions could interfere with important enzymatic reactions in brain cells involved in the synthesis, release, or degradation of neuronal transmitter substances.

In general, the results reported here indicate that the phenomenon of general anesthesia may be associated with an effect of the anesthetic agent on calcium movements in the cell. Our observations give support to the original suggestion of Heilbrunn\textsuperscript{21, 22} that suppression of neuronal activity by an excess of calcium ions may occur during anesthesia. Halothane certainly has the ability to interfere with calcium transport by brain mitochondria. Whether this phenomenon plays a role in the production of anesthesia in the whole organism is not clear at present, but is a problem of considerable importance.

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References


