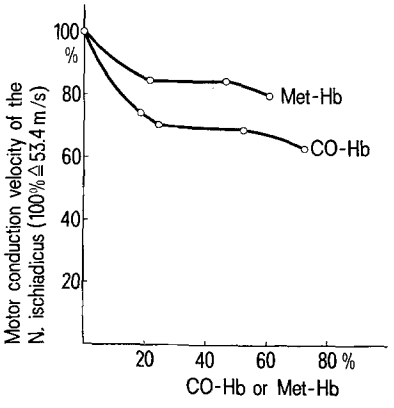
Results and discussion. The alteration of the motor conduction velocity depends on the impairment of hemoglobin function. Increasing carboxy- or methemoglobinemia to about 20% produces a distinct decrease, then to about 50% only insignificant changes and final a farther decrease of the motor conduction velocity (Figure).



Motor conduction velocity of the N. ischiadicus of rats following single injections of 0.5, 0.8, 2.4 and 12 mmol CO/kg (s.c.) or 0.4, 0.8 and 1.2 mmol $NaNO_2$/kg (i.p.).

But the extent of the retardation of nerve conduction in hypoxic conditions of the same degree is about twice as high following CO than $NaNO_2$ intoxication. This means that CO produced an additional effect on peripheral nerve function beside the hypoxic response induced by blockade of hemoglobin. A significant ($p < 0.001$) decrease of motor conduction velocity was observed already if a mean carboxyhemoglobinemia of 18.6% CO-Hb or in particular case of 13% CO-Hb was determined. It seems that the motor conduction velocity is a sensitive indicator for testing peripheral nerve after CO intoxication.

Zusammenfassung. Störung der Sauerstofftransportfunktion des Hämoglobins durch Carboxy- oder Methämoglobinämie vermindert die motorische Leitungsgeschwindigkeit am N. ischiadicus der Ratte. Das Ausmass der Verlangsamung der Nervenleitung ist nach gleichgrosser Blockade des Hämoglobins unter Kohlenmonoxideinfluss annähernd doppelt so gross wie unter Natriumnitriteinfluss.

D. PANKOW, W. GLATZEL, I. GRIMM, W. PONSOLD and K. TIETZE

Lehrstuhl für Industrietoxikologie am Hygiene-Institut und Klinik und Poliklinik für Psychiatrie und Neurologie der Martin-Luther-Universität, Leninallee 4, DDR–402 Halle a.d. Saale (GDR), 22 March 1974.

Ethyl-m-Aminobenzoate (MS-222) Anaesthesia in the Newt - Effect of D_2O, pH and Time of Day

Ethyl-m-aminobenzoate (metacain, MS-222) is a much used anaesthetic for cold blooded animals[1]. Although its mechanism of action is unknown, one likely possibility is that it inhibits transmitter release[2], and a reciprocal antagonism between ethyl-m-aminobenzoate and serotonin in several invertebrate species has been observed[3]. Recently it has been reported[4] that the rate of anaesthesia of the fish *Mollensia spec.* by various doses of MS-222 decreased markedly if 20–40% D_2O was substituted for water in the aquarium, and the results were attributed to the membrane-stabilizing properties of D_2O.

This interpretation is at variance with current theories of anaesthesia[5,6], and we have therefore examined the effect of D_2O on anaesthesia in aquatic amphibia. The urodeles, newts and salamanders, are particularly suitable for studies of this nature since metamorphic changes permit the examination of both water-and air-breathing forms. Further, the use of an aquatic animal and a water-soluble anaesthetic such as MS 222 permit the control of critical factors involved in the structure of water, which has been implicated in anaesthesia[5,6].

Materials. Ethy-m-aminobenzoate (MS 222) was obtained from Sandoz, Ltd. Basle, Switzerland. D_2O was obtained from the Isotope Separation plant of the Weizmann Institute (as a by-product of oxygen isotope separation). The protium content of this heavy water was determined by nuclear magnetic resonance and the deuterium content obtained by difference. The deuterium content ranged from 40%–97% and was diluted as described below.

Animals. Adult newts (*Triturus cristatus*) were previously maintained in a temperature-controlled room (22 ± 2°C) under constant light with continuous aeration for at least 6 months prior to the experiment. Larvae of the newt *Pleurodeles waltl* were raised in the laboratory from eggs under the above conditions and were 80–100 days old when used. The parthenogenic fish *Poecillia formosa* were obtained from Gossington Tropical Fisheries, Box 208, Delray Beach, Florida, and were maintained for at least 12 months in the same temperature-controlled room with continuous aeration and constant light.

Table I. Effect of D_2O on ethyl-m-aminobenzoate (MS 222) anaesthesia in the adult newt (*Triturus cristatus*)

	Anaesthesia time (sec)	Activity	Escapes
H_2O	–	7.2 ± 15.0	6.3 ± 15.0
H_2O + MS 222	747 ± 98	18.3 ± 5.8[a]	56.3 ± 38.7[a]
25% D_2O + MS 222	718 + 103	20.3 ± 7.0[a]	43.5 ± 20.7[a]

9 adult *Triturus cristatus* (6.2–9.4 g), raised in constant light and kept in Rehovot tapwater (RST) for 24 h before use. Activity and escape behaviour were determined during 5 min after transfer to a control tank of RST and during the first 5 min after subsequent transfer to freshly prepared solutions of MS 222 (133.3 mg/l). Each animal was tested twice in each experimental solution and the average time for anaesthesia in each condition was used to compute the group means expressed as mean ± S.D. [a] Indicates a significant difference from control, $p < 0.05$.

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Methods. In all experiments the water has the same ionic composition as Rehovot standard tapwater (RST), in mEq/l: 2.33 Na, 0.60 K, 0.74 Ca, 0.585 Mg, 3.82 Cl, 0.08 SO_4 and 0.38 HCO_3. This composition was based upon existing analytical data on the local water supply. The pH of RST is 5.74 and that of the H_2O-D_2O mixture adjusted to 5.84 in accordance with the rule pD = pH + 0.4[7]. Stock solutions of MS 222 were prepared immediately before each experiment and were discarded immediately after use. The MS 222 concentrations used are given in the text. The temperature and the pH of the solutions were determined before and after each experiment. Statistical comparisons were carried out using a Student *t*-test.

Table II. Effect of D_2O on ethyl-m-aminobenzoate (MS 222) anaesthesia in the newt larvae (*Pleurodeles waltl*)

	Pretreatment	Dose (mg/l)	Anaesthesia time (sec)
H_2O	—	50	311 ± 98 (10)
40% D_2O	—	50	177 ± 40[a] (10)
H_2O	H_2O	75	263 ± 27 (10)
25% D_2O	25% D_2O	75	221 ± 52[a] (11)

80- to 100-day-old *Pleuradeles* larvae reared in constant light were maintained in RST for 24 h before transfer to beakers containing freshly prepared MS 222 solutions. The results are expressed as mean ± S.D. Number of animals in parentheses. [a] Indicates a significant difference from control $p < 0.05$.

Table III. Effect of D_2O on ethyl-m-aminobenzoate (MS 222) anaesthesia in the fish (*Poecilia formosa*)

	Anaesthesia time (sec)
H_2O	326 ± 136 (6)
25% D_2O	232 ± 75 (5)

The fish were raised under constant light and kept in RST for 24 h before testing. They were transferred to beakers containing freshly prepared solutions of MS 222. The results are expressed as means ± S.D. The number of animals in parentheses.

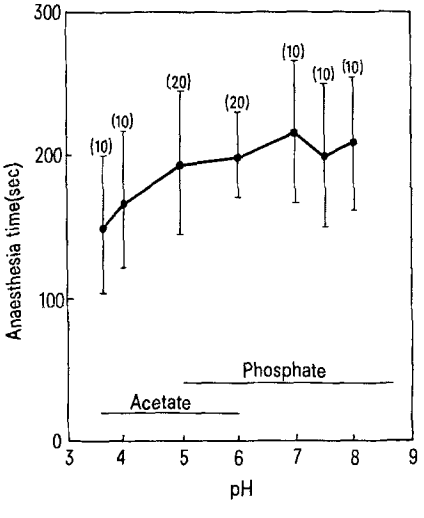

Fig. 1. Effect of hydrogen ion concentrations on ethyl-m-aminobenzoate (MS 222) anaesthesia in newt larvae (*Pleurodeles waltl*). 120-day old *Pleurodeles* larvae reared in constant light were taken from aquarium water and anaesthetized in freshly prepared 0.005 *M* sodium acetate or 0.005 *M* sodium phosphate buffers containing MS 222 (75 mg/l). The number of animals is indicated in parentheses on each point. The error bars indicate the standard deviation of the mean.

Testing. Adult animals were tested in opaque, white, plastic (19.7 × 10 × 10 cm) containers with 500 ml of either RST or a mixture of 25% D_2O – 75% H_2O. Animals were taken from their home tanks, dried on paper towelling and placed in a control container with fresh RST for 5 min, and then transferred to the test container. Activity was determined from the number of passages of the head over 3 equidistant lines marked on the floor of the containers, and the number of 'escapes' in which the head was raised to the top of the tank. Newts exposed to MS 222 showed an initial increase in movement and in escape responses, which is common to many anaesthetics in many species. Each adult newt was tested for anaesthesia by being periodically turned on its back in the water until it remained in this position for 30 sec. The time, from the entry of the animal into the container to the beginning of this 30-sec period, was taken as the anaesthesia time. Each animal was tested twice in D_2O and twice in H_2O in alternating order, with a minimum of 3 h between tests.

The criteria for anaesthesia in the fish was the complete absence of tail or pectoral fin movements in the water, and absence of response on being lifted with a wire loop (8 cm in diameter) supporting a tightly stretched nylon net out of, and then returned to, a beaker containing the anaesthetic solution. The onset of anaesthesia in newt larvae was determined by lifting the animal from the water 3 times, for 5 sec in immediate succession without eliciting an escape response or other movements.

Results. The effect of various deuterium concentrations on anaesthesia time, activity and escapes of adult and larval newts and in parthenogenic fish are given in Tables I, II and III, respectively. It is evident that deuterium increases the rate of anaesthesia in all three groups. The trend is obvious but only in larval newts is the difference statistically significant. This result is contrary to that reported for the fish, *Mollensia spec*[4]. Changes in MS 222 concentration or pretreatment (Table II) did not affect the direction of our results.

The effect of pH on MS 222 anaesthesia was also examined (Figure 1). Since MS 222 is a primary amine salt of methanesulfonic acid, in distilled deionized water it dissolves to give a distinctly acid solution. For this reason the pH was adjusted in all experiments to compensate for this acidity. Figure 2 shows the titration curve of the preparation of MS 222 used in these studies. The inflection point near pH 3.5 corresponds to the ionisation of the sulfonic acid, but that of the primary amine group does not appear under these conditions. As seen in Figure 1 the rate of anaesthesia is pH sensitive.

Since the pharmacological properties of many agents show quantitative variations at different times of the day, the rate of anaesthesia at various times was also studied (Figure 3).

Discussion. Before a specific mechanism for effect of D_2O on anaesthesia can be suggested, more detailed information on its effect on MS 222 absorption, distribution and metabolism and on the mechanism of anaesthesia are required. In general, the anaesthetic properties of a number of unreactive molecules, including Xenon, have been explained on the basis of clathrate formation[5],

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causing stabilization of intra-neuronal water and hence increased rigidity of neuronal structures. It has been pointed out that water at intracellular surfaces closely resembles ice in structure[8], and that ice-like structure persists at higher temperatures for D_2O than for water as witnessed by the marked differences in temperature-density profiles[9]. Hence, an increased D_2O content might be expected to stabilize the intracellular water at or near membranes in the same manner as clathrate-forming compounds and should, therefore, potentiate anaesthetics operating by this mechanism. The present results of an increased rate of anaesthesia in D_2O are consistent with such an explanation.

A number of other points of interest emerge from this study. First, a marked difference exists in species sensitivity to MS 222 anaesthesia. The adult newt seems relatively resistant to MS 222, taking nearly 500 sec for anaesthesia whereas in *Pleurodeles* larvae it was too rapid to be measurable by our techniques and took 28 sec in the fish *Poecilla formosa*, all at the same concentration (535 mg/l). The relative insensitivity of the newt may, however, be due to slower MS 222 absorption through the skin or lungs, in contrast to absorption through the gills.

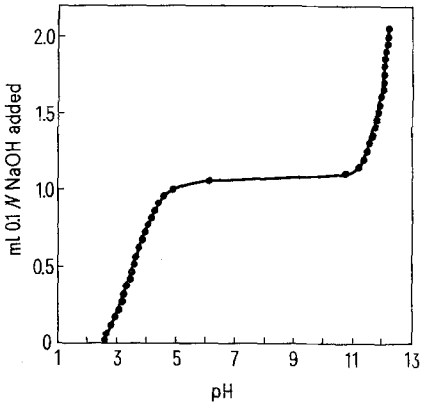

Fig. 2. Titration curve for ethyl-m-aminobenzoate methane-sulfonate (MS 222). The initial solution containing MS 222 (100 mg) in 100 ml of distilled deionized water (3.8×10^{-2} *M*) was neutralized with 0.1 *N* NaOH.

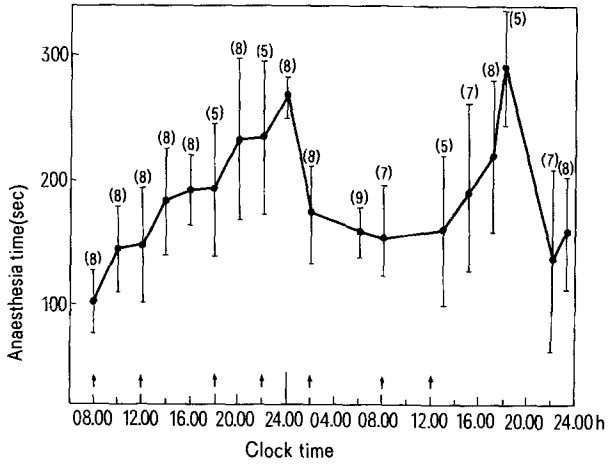

Fig. 3. Diurnal variations in the ethyl-m-aminobenzoate (MS 222) anaesthesia in newt larvae (*Pleurodeles waltl*). 80–100-day-old *Pleurodeles* larvae reared in constant light were kept in 0.005 *M* sodium phosphate buffer, pH 7.0 for 18 h. At the times indicated above, the rate of MS 222 (75 mg/l) anaesthesia in 0.005 *M* sodium phosphate buffer or 0.005 *M* sodium acetate buffer was determined. Fresh buffer – MS 222 solutions were prepared at the times indicated by the arrows. The number of animals are indicated in parentheses at each point. The error bars indicate the standard deviation of the mean.

It is well known that the pharmacological effects of many agents, including anaesthetics, show variations with time of day due perhaps to changes in receptor sensitivity or in liver microsomal drug-metabolizing enzymes. The marked ciradian rhythm, found in MS 222 anaesthesia in *Pleurodeles* larvae (Figure 3), appears to be free running and ultradian with a periodicity less than 24 h. These results reinforce the importance of controlling the time of treatment in pharmacological studies generally, and in studies on anaesthetics in particular. The present results also show a decrease in the rate of anaesthesia at low pH. The titration curve for MS 222 was relatively flat between pH 5–8 so that the observed effect on anaesthesia probably reflects the pH sensitivity of biological processes, such as transport and metabolism, rather than differences in ionic species of MS 222. In D_2O solutions, this effect may be even larger since pH and pD are not equal[7].

In addition to the effect of deuterium on dissociation constants, there are several other possible explanations for the discrepancy between our results and those[4] in another species of fish. It is known that D_2O decreases respiration of cortical tissues in vivo[10,11] and also that O_2 and CO_2 are 10% less soluble in D_2O than in H_2O[12]. If, in some fish, MS 222 anaesthesia is related to oxygenation, this difference in solubility and respiration may result in apparent differences in anaesthesia sensitivity. It is also possible that, since MS 222 is an ester, it is sensitive to both acid and alkaline hydrolysis, which is known to be affected by the deuterium content of the medium[13]. The deuterium in the medium may, therefore, affect the rate of anaesthesia by altering the ester (MS 222) concentration. Finally, D_2O has been reported to alter the circadian phasing of the mouse[14] and *Phaseolus*[15] and conceivably might also shift circadian phasing in the fish. A desynchrony between water and D_2O-treated fish might appear as a difference in MS 222 sensitivity. We are in contact with Dr. WENZEL in order to find ways of resolving this conflict.

Résumé. Des concentrations de deutérium de l'ordre de 25–40% dans le milieu, ont légèrement augmenté la vitesse de l'anaesthésie produite par le m-aminobenzoate d'éthyle (MS 222) sur les larves et les adultes d'urodèles et sur une espèce de poisson. On a observé que cette vitesse a un rhythme circadien, et a été influencée par le pH. Ceci est compatible avec les théories actuelles sur les effets des anaesthésiques sur la structure de l'eau dans le système nerveux central.

A. YUWILER[16] and D. SAMUEL

Isotope Department, Weizmann Institute of Science, Rehovot (Israel),
15 February 1974.

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[16] Visiting scientist from the Neurobiochemistry Laboratory, T-85, Veterans Administration Hospital, Wilshire and Sawtelle Blvds., Los Angeles, California, and the Dept. of Psychiatry, University of California at Los Angeles Medical School, Los Angeles, USA.