Beneficial Effect of Fluorocarbon Emulsion Media on the Function of Neuromuscular Preparations in Vitro

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ABSTRACT The effects of liquid fluorocarbons as bathing media were determined by use of in vitro neuromuscular preparations. Rat hemidiaphragms were bathed in either oxygenated fluorocarbon (FC) emulsion or standard oxygenated Krebs solution. Contractile force in response to simple supramaximal nerve stimuli as well as to high frequency stimulation was greater, while twitch:tetanus ratio was smaller in FC emulsion. With such medium, post-tetanic potentiation of contraction was also more consistently observed. Indirectly stimulated diaphragms survived longer in FC emulsion. After cessation of oxygenation, oxygen tension (pO₂) of the medium declined more rapidly with Krebs than with FC emulsion; pO₂ directly correlated with force of contraction. Similarly, in the chick biventer cervicis preparation, FC emulsion enhanced nerve-stimulated force of contraction; returning the preparation to standard Krebs solution reversed this phenomenon. Dose-response curves of muscle contraction in response to acetylcholine and KCl administration were shifted upward during FC emulsion superfusion. Frequency of miniature endplate potentials was lower in FC emulsion than that observed in Krebs solution, measured from the same cell of the rat diaphragm. Resting membrane potentials were also greater in muscle cells sampled from FC emulsion-bathed preparations. These data suggest that FC emulsion is superior to standard Krebs solution as a bathing medium for in vitro neuromuscular preparations by virtue of the high solubility of oxygen in it.

INTRODUCTION

In vitro preparations have been employed in the majority of studies of electrophysiological events and pharmacological effects of the mammalian neuromuscular junction (NMJ). However, investigations utilizing in vitro preparations have shown discrepancies with regard to several indices used to measure neuromuscular functions in vivo. For example, miniature endplate potentials (MEPPs) recorded from in vivo preparations have lower frequencies than those observed with in vitro preparations (Muchnik, 1975). In addition, in vivo MEPPs have larger amplitudes, faster rates of rise, and shorter durations than those recorded in vitro. It has been suggested (Hoekman et al., 1974; Muchnik, 1975) that these differences between in vitro and in vivo effects might be attributed to the relative hypoxic condition of in vitro systems.
Hubbard and Loyning (1966) observed a progressive increase in MEPP frequency accompanied by a loss of resting membrane potential (RMP) in rat phrenic nerve diaphragm deprived of oxygen. Hypoxic cells have also been shown to lose potassium and gain sodium (Creese, 1954) which correlates with a diminution of endplate potential (EPP) amplitude (Hubbard and Loyning, 1966) and force of contraction (Paul, 1961).

Numerous studies (Creese et al., 1958; Paul, 1961; Hubbard and Loyning, 1966) have shown that electrophysiological events at the neuromuscular junction are easily altered by slight differences in the degree of oxygenation. In vitro preparations are bathed in a physiological salt solution and bubbled with oxygen. Under the usual experimental conditions, these salt solutions can hold only one-seventh as much oxygen as blood (Clark et al., 1972). This suggests that the oxygen supply to the neuromuscular junction in in vitro preparations may be inadequate. Recently, a group of liquid fluorocarbons with high oxygen solubility (50 vol %) developed by the 3M Co. (St. Paul, Minn.) have become available for biological research. An emulsion consisting of 30–40% fluorocarbon chemicals in a physiological saline solution can hold as much oxygen as blood (Clark et al., 1972).

The purpose of this investigation was to determine whether fluorocarbon emulsion might be more suitable as a bathing medium than Krebs solution for in vitro studies of neuromuscular junction by virtue of its superior oxygen-carrying capacity.

MATERIALS AND METHODS

Preparation of Fluorocarbon Emulsion

Fluorocarbon emulsions were prepared by sonicating a mixture of 20% fluorocarbon (FC 80) and 80% Krebs solution (composition in grams per liter of distilled water: NaCl, 5.5; KCl, 0.35; MgSO₄·7H₂O, 0.29; CaCl₂, 0.28; KH₂PO₄, 0.16; NaHCO₃, 2.1; dextrose, 4.0). Pluronic F68 (Wyandotte Co., Mich.) (150 mg/100 cm²) was added as the emulsifying agent. Sonication with a Biosonic IV (Bronwill, Rochester, N. Y.) sonicator for 20 min was usually sufficient to generate a homogeneous emulsion. Additional brief (5 min) periods of sonication were used when necessary. This emulsion was placed in a water bath at 36–38°C and bubbled with a mixture of 95% O₂, 5% CO₂. Total oxygen content of the Krebs solution or the fluorocarbon emulsion was measured with a Lex-O₂-Con oxygen analyzer (Lexington Instruments Corp., Waltham, Mass.). Oxygen tension was measured with a pO₂ electrode, type E 5046, acid-base analyzer, type PHM 71 Radiometer.

In vitro Preparations

RAT PHRENIC NERVE-DIAPHRAGM PREPARATIONS Male Sprague-Dawley rats (80–150 g) were used. The method employed was that described by Bülbring (1946). In brief, this involved decapitation of the animal with subsequent opening of the thorax. The left phrenic nerve was isolated and removed with the left hemidiaphragm. These tissues were mounted on a muscle holder equipped with platinum electrodes over which the phrenic nerve was carefully placed. After attachment of the suture to the central tendon, the holder and mounted diaphragm were placed in an organ bath containing either 50 ml of Krebs solution or 50 ml of fluorocarbon emulsion. Both solutions were maintained at 37°C and bubbled with 95% O₂, 5% CO₂. The suture was attached to a force-displacement transducer and an initial tension of 5 g was placed on the muscle. The nerve was then
stimulated with rectangular pulses at 0.2 ms duration at a frequency of 0.2 Hz except during the period of tetanic stimulation. The frequency and duration of the latter are indicated in Results. Supramaximal stimuli (15-25 V) were used throughout.

The following measurements were made: force of contraction, tetanus height, and twitch:tetanus ratio. In addition, the frequency of stimulation (fusion frequency) that will produce a fused tetanus of muscle contraction was measured. Post-tetanic potentiation (PTP) of muscle contraction after high frequency stimulation was also measured and expressed as percent of control. Longevity of the preparations at 0.2 Hz and 1 Hz was measured in terms of the time that elapsed from the initial muscle twitch in response to supramaximal nerve stimulation to the time at which the muscle would no longer contract in response to the same intensity of nerve stimulation. The time to reduction of contraction to 50% of control at 2 Hz and at 4 Hz was also measured.

**CHICK BIVENTER CERVICIS NERVE MUSCLE PREPARATION**

1-2-wk old chicks were killed with chloroform. The biventer cervicis muscle was isolated according to the method of Ginsborg and Warriner (1960). The lower belly of the muscle was then attached to a stationary rod and the upper belly to a force-displacement transducer, and an initial tension of 1 g was placed on the muscle. The muscle was then superfused with either Krebs solution or fluorocarbon emulsion warmed to 37°C and bubbled with 95% O₂-5% CO₂. The motor nerve within the tendon was stimulated electrically at a frequency of 0.1 Hz, with a pulse duration of 0.1 ms at supramaximal voltage (20-30 V). The force of contraction of the preparation was measured when either the Krebs solution or fluorocarbon emulsion was used as superfusion medium.

The chick biventer cervicis nerve muscle preparation was also used to examine the effect of fluorocarbon emulsion as superfusion medium on the response to acetylcholine and potassium chloride. Acetylcholine and potassium chloride were administrated by injection into the superfusate just above the muscle.

**MICROELECTRODE STUDIES**

All experiments were carried out in vitro at a temperature of 36-38°C with the isolated rat phrenic nerve diaphragm. The preparation was mounted in a lucite bath of 3 ml vol on a paraffin-lined Plexiglass plate in the center of which was a planoconvex lens. The muscle was constantly perfused (flow rate 6 mg/min) with either Krebs solution or fluorocarbon emulsion. Intracellular potentials were measured with glass capillary microelectrodes of 8-15 MΩ resistance filled with 3 M KCl in a conventional manner similar to that employed by Akerman and Sokoll (1969). Miniature endplate potentials (MEPPs) and the resting membrane potential (RMP) of the cells were monitored. Endplate regions were located by finding MEPPs with a rapid rising phase. The potentials were recorded and stored on magnetic tape for subsequent analysis using a PDP-11 computer. The following measurements were made for each perfusion medium: the resting membrane potential (RMP) of the muscle cell, and the frequency, rate of rise, duration, and amplitude of MEPPs.

**Statistics**

Comparisons of sample means were made, employing a group t-test or a paired t-test. For multiple dose-response relationships, an analysis of variance: factorial design was employed. Best-fitting lines for dose-response curves were determined by regression analysis. In all cases, a P value of <0.05 was considered significant.

**RESULTS**

**Oxygen-Carrying Capabilities of the Fluorocarbon Emulsion**

Krebs solution which was bubbled for 1 h with 95% O₂-5% CO₂ was found to contain 2.29 ± 0.03% (SE) oxygen by volume with an oxygen tension of 690.0 ±
11.6 mm Hg. In contrast, a 20% fluorocarbon emulsion was found to contain 8.03 ± 0.44% oxygen by volume with an oxygen tension of 711.6 ± 9.29 mm Hg. When oxygenation of Krebs solution was terminated, the oxygen content declined to a level of 1.43 ± 0.03 vol% in 60 min. In comparison, the 20% fluorocarbon emulsion maintained a higher oxygen content of 7.53 ± 0.41 vol% within the same period. The rate of decline in oxygen tension of these two media is depicted in Fig. 1.

**FIGURE 1.** A comparison of the decline of oxygen content and oxygen tension in normal Krebs solution vs. a 20% fluorocarbon emulsion. Values = mean ± SE; n = 3.

* Rat Phrenic Nerve-Diaphragm Preparations

Measurement of contraction characteristics of in vitro rat phrenic nerve diaphragm preparations were made for both bathing media: the standard Krebs solution and the fluorocarbon emulsion, respectively.

The force of contraction, tetanus height, twitch:tetanus ratio of diaphragm, and the fusion frequency of stimulation measured when Krebs solution was used as bathing medium are shown in Table I. With Krebs solution as the medium, only 4 out of 10 of the preparations responded to high frequency stimulation (100 Hz, 10 s) with a post-tetanus potentiation (PTP) of contraction (Table I).

Longevity of the preparations bathed in Krebs solution and stimulated at 0.2 Hz and at 1 Hz is given in Table I. There was no difference in the longevity of preparations bathed in Krebs solution stimulated at 0.2 Hz and at 1 Hz. Time to reduction of contraction to 50% of control at 2 Hz and 4 Hz is also shown in Table I.
When fluorocarbon emulsion was used as the bathing medium, the force of contraction and tetanus strength of the diaphragm were significantly increased (Table I). Twitch:tetanus ratio was lower than that obtained when Krebs solution was used as bathing medium (Table I). In addition, with fluorocarbon emulsion as bathing medium, each of the muscle preparations responded to higher frequency stimulation (100 Hz, 10 s) with PTP. A typical PTP is shown in Fig. 2.

**Table I**

|                          | Krebs solution | Fluorocarbon emulsion (FC80) (20%) |
|--------------------------|---------------|-------------------------------------|
| Force of contraction (g) | 14.10±0.92\*  | 17.00±0.83\‡                       |
| Tetanus height (g)       | 37.2±2.75     | 51.2±3.04\‡                       |
| Twitch/tetanus ratio     | 0.38±0.02     | 0.34±0.01\†                        |
| Fusion frequency (Hz)    | 34.4±1.51     | 36.0±1.23                          |
| Post-tetanic potentiation (PTP) (% of control) | 117±8.0 | 149±6.5\‡                      |
| Longevity at 0.2 Hz (min) | 157±36       | 508±79\‡                           |
| Longevity at 1 Hz (min)  | 105±34        | 291±48\‡                           |
| Time to reduction of contraction to 50% of control at 2 Hz (min) | 6.9±1.7 | 23.9±4.1\‡                     |
| Time to reduction of contraction to 50% of control at 4 Hz (min) | 1.3±0.5 | 7.9±2.9\‡                       |

* Value = mean ± SE n = 10.
\‡ Denotes significant difference, group t-test P < 0.05.

The PTPs measured were consistently and reproducibly generated in all preparations and had a higher magnitude than that obtained in preparations bathed in Krebs solution (Table I). The only measure that was not demonstrated to be significantly altered when fluorocarbon emulsion was used instead of standard Krebs solution was the fusion frequency.

A further series of experiments using rat phrenic nerve diaphragm was undertaken to verify that the force of contraction developed by muscle was greater in fluorocarbon emulsion than in Krebs solution. These experiments
were performed in the paraffin-layered well originally designed for microelectrode studies. The preparations were first perfused with Krebs solution and subjected to supramaximal stimulation. The perfusion medium was then changed to fluorocarbon emulsion after a steady state of contraction was reached. In all five of the preparations, the force of contraction was greatly enhanced during perfusion of fluorocarbon emulsion. A typical record is shown in Fig. 3. The average force of contraction was 5.6 ± 1.59 g (SE, n = 5) in Krebs solution and 11.7 ± 0.87 g (n = 5) in fluorocarbon emulsion. Neither the shape nor the duration of muscle contraction differed in both perfusion media. When the preparations were perfused by Krebs solution, no PTP occurred after a high-frequency stimulus (100 Hz) was interposed for a period of 10 s. In contrast, the PTP was produced in the same diaphragm under the same condition as when fluorocarbon emulsion was used for perfusion.

![Figure 3](image.png)

**Figure 3.** Typical record of the effect of a 20% fluorocarbon emulsion on the force of contraction of the electrically stimulated rat phrenic nerve diaphragm preparation. The nerve was stimulated supramaximally at 0.2 Hz. At the arrow the perfusion medium was changed from normal Krebs solution to a 20% fluorocarbon emulsion.

The following experiments were undertaken in order to correlate the oxygen content and the oxygen tension of the bathing medium with the activities of the muscle preparations. Diaphragms were placed in a chamber containing either Krebs solution or FC emulsion which had been previously oxygenated. The diaphragms were either stimulated through the nerve at 0.2 Hz or not stimulated. The decline of the oxygen content and oxygen tension were measured over a period of 60 min. In both conditions, the oxygen content and oxygen tension of Krebs solution declined to a much lower level than that observed with the fluorocarbon emulsion over period of 1 h (Fig. 4).

For example, when the muscle was indirectly stimulated (0.2 Hz), the oxygen content for Krebs solution was 0.40 ± 0.06 vol % (SE) and its \( pO_2 \) was 153.8 ± 26.4 mm Hg after 60 min. In comparison, under similar conditions, the oxygen content for FC emulsion was 4.97 ± 0.56 vol % and its \( pO_2 \) was 477.5 ± 29.5 mm Hg. When muscle contraction was measured in preparations with nerve stimulation, a direct correlation between oxygen tension of either bathing medium and the force of contraction was observed (Fig. 5). Force of contraction of the diaphragm decreased with the decline of oxygen tension in both media. After 1 h, fluorocarbon emulsion was again bubbled with 95% \( O_2 \)-5% \( CO_2 \). Force of contraction increased to its initial value as oxygen tension of the bathing medium increased. A similar but less complete recovery of muscle contraction was also observed upon reoxygenation of Krebs solution.
Without emulsification, neither Pluronic F68 alone nor the fluorocarbon chemicals had any effect on the indices measured.

**Chick Biventer Cervicis Nerve Muscle Preparation**

When the superfusion medium of the chick biventer cervicis preparations was changed from Krebs solution to fluorocarbon emulsion, the force of contraction of the muscle increased. The average force of contraction of a muscle superfused with Krebs solution was 1.72 ± 0.42 g, while of that superfused with fluorocarbon emulsion it was 2.35 ± 0.56 g. The force of contraction remained at a significantly higher level as long as the preparation was superfused by fluoro-
carbon emulsion. When Krebs solution was used again as a superfusion medium, the force of contraction returned to its original lower value.

Similar results were obtained upon the direct injection of either acetylcholine (ACh) or potassium chloride to the muscle. The dose-response curve of ACh on muscle contraction was shifted by a factor of 2.7 when fluorocarbon emulsion was the superfusion medium. For example, in Krebs solution the injection of 3 μg of ACh produced a contraction of 0.42 ± 0.05 g. In fluorocarbon emulsion, it was increased to 1.24 ± 0.60 g. In a similar fashion, the dose-response curve of potassium chloride on muscle contraction was shifted by a factor of 2.0 (Fig. 6). Again, the injection of 3 μg of KCl produced a contraction of 0.18 ± 0.03 g in Krebs solution and 0.56 ± 0.14 g in fluorocarbon emulsion.

Figure 5. A correlation between force of contraction of in vitro rat phrenic nerve diaphragm preparation and oxygen tension (pO₂) of the bathing medium. Values = mean, n = 4. ▲ Represents measurements made when Krebs solution was used as bathing medium. ● Represents measurements made when fluorocarbon emulsion was used as bathing medium. ○ Represents measurements made when force of contraction reached 50% of control in all preparations.

Neither the emulsifying agent Pluronic F68 nor the fluorocarbon emulsion had an effect of its own on the indices measured.

Microelectrode Studies

The effect of fluorocarbon emulsion on an index of spontaneous transmitter release was also investigated. Amplitude, rate of rise, and duration of MEPPs were not significantly altered when the perfusion medium was changed to the fluorocarbon emulsion. However, the MEPPs frequency was significantly reduced (Table II). In addition, in 6 of the 13 cells, RMP increased substantially by an average value of −8 mV when the perfusion medium was changed from Krebs solution to fluorocarbon emulsion. The remaining cells retained a similar magnitude of RMP in both perfusion media.

To investigate further the hyperpolarizing effect of fluorocarbon emulsion on RMP of muscle cells, RMP of 100 randomly selected cells, perfused by either Krebs solution or fluorocarbon emulsion, were measured. The average RMP of
cells perfused by fluorocarbon emulsion was $-78.16 \pm 1.06$ mV and RMP of cells perfused by Krebs solution was $-73.28 \pm 0.97$ mV. The difference of $-5$ mV was significant ($P < 0.05$).

![Figure 6](image)

**Figure 6.** The effects of ACh and potassium chloride on muscle contraction of the chick biventer cervicis, comparing normal Krebs solution with fluorocarbon emulsion as the bathing medium. The lines represent the best fitting line as calculated by linear regression. Values = mean ± SE; $n = 7$; $P < 0.05$.

|                         | Fluorocarbon Emulsion (FC80 (20%)) | Krebs Control |
|-------------------------|-----------------------------------|---------------|
| Frequency (MEPPs/s)     | 2.85±0.55*                        | 1.93±0.35‡    |
| Amplitude (mV)          | 0.74±0.05                         | 0.71±0.05     |
| Rate of rise (V/s)      | 1.05±0.06                         | 1.01±0.06     |
| Duration (ms)           | 2.55±0.13                         | 2.74±0.12     |

* Value = mean ± SE, $n = 13$.
‡ Denotes significant difference, paired t-test ($P < 0.05$).

**DISCUSSION**

The results of this investigation suggest that fluorocarbon emulsion is a better bathing medium than standard Krebs solution for in vitro mammalian neuromuscular preparations. When fluorocarbon emulsion was employed as the bathing medium for isolated neuromuscular preparations, contraction characteristics such as force of contraction, tetanus height, twitch:tetanus ratio, and occurrence of PTP were all improved. Longevity of all the preparations was also increased when fluorocarbon emulsion was used. The ability of fluorocarbon emulsion to deliver a greater amount of oxygen to the tissue would appear to be the explanation for this improvement in muscle function.
As noted by other investigators (Clark and Gollan, 1966; Gollan and Clark, 1966; Geyer, 1973) and confirmed by the data of our present experiments, the solubility of oxygen in liquid fluorocarbon was high. A 20% fluorocarbon emulsion was found to dissolve about three times the amount of oxygen compared to the standard Krebs solution. In all probability, a higher oxygen content of fluorocarbon emulsion may serve as a reservoir of oxygen which helps to maintain an optimal oxygen tension in the bathing medium. This conceivably results in a greater diffusion gradient of oxygen between the tissue and the medium. Thus the high oxygen demand of the preparation is met. Such a hypothesis is supported by our present finding of both a direct correlation between oxygen tension (pO₂) of the medium and the functional state of the muscle, and by the observation that the fluorocarbon emulsion maintains a higher pO₂ for a longer period than the Krebs solution.

When fluorocarbon emulsion replaced Krebs solution as the perfusion medium in our experiments, the force of contraction of the preparations increased dramatically. Such a response is similar in nature to observations of the effect of hypoxia on muscle function made by other investigators (Paul, 1961; Hubbard and Loyning, 1966). They reported that the force of contraction declined rapidly when the muscle was subjected to a hypoxic condition, and upon reoxygenation there was a rapid recovery of contraction strength. One of the primary effects of hypoxia is the interference of the normal aerobic metabolism of the tissue (Calkins et al., 1954) which reduces the efficiency of the excitation-contraction coupling of the muscle. Therefore it is quite conceivable that better oxygenation by fluorocarbon emulsion causes an increase in force of contraction of muscle in response to nerve stimulation.

The decrease of twitch:tetanus ratio in fluorocarbon emulsion may be explained in terms of Hill's concept of the active state (Hill, 1949, 1950). It appears that better oxygenation may result in a shortened duration of the plateau of the active state of the contractile elements of the muscle. Likewise, the increased tetanic contraction strength in response to high-frequency stimulation may also reflect an increase in the intensity of the active state. Together these may serve as an indication of an improvement in the metabolic state of the muscle due to better oxygenation of the preparation by use of our new bathing medium. Such improvement appeared to be confirmed by the prolonged survival time of the preparations.

In vitro neuromuscular preparations do not have the ability to respond to high-frequency stimulation for long periods (Brooks and Thies, 1962). With high-frequency stimulation, both the force of contraction and the amount of ACh released per impulse drop rapidly (Straughan, 1960; Krnjevic and Mitchell, 1961; Elmqvist and Quastel, 1965). In addition, most attempts to produce post-tetanic potentiation (PTP) of muscle contraction in in vitro neuromuscular preparation bathed in Krebs solution via high-frequency stimulation led to rapid deterioration of the preparation. This is in contrast to in vivo preparations where high-frequency nerve stimulation results in the PTP of muscle contraction (Standaert, 1964). In the present study, PTP could be readily and consistently observed in vitro when fluorocarbon emulsion was used as the bathing medium.
In the present in vitro experiments, rat diaphragms bathed in fluorocarbon emulsion demonstrated an average resting membrane potential (RMP) of 78.2 ± 1.0 mV which was higher than that (73 mV) reported in other comparable in vitro studies (Liley, 1956). Creese et al. (1958) reported that the lack of oxygen characteristically produces lower RMP. Hence, our finding of higher RMP supports the contention that by the use of a fluorocarbon emulsion in vitro neuromuscular preparation results in a better-oxygenated preparation.

The hyperpolarizing effect of optimal oxygenation provided by fluorocarbon emulsion may be a general membrane phenomenon, not limited solely to the muscle membrane. Thus, the hyperpolarization of nerve terminal may explain the lower MEPPs frequency observed in this investigation. Other investigations have reported that frequency of MEPPs is lower in vivo than in vitro (Muchnik et al., 1975). This has been explained on the basis of difference in the oxygenation of the preparation (Hoekman et al., 1974). Conversely, under hypoxic conditions, the depolarization of nerve terminal increased frequency of MEPPs (Hubbard and Loyning, 1966). Our present finding suggests that frequency of MEPPs may be a good criterion of oxygenation of the in vitro preparation.

Data derived from in vitro studies of synaptic function at the neuromuscular junction have played an important role in the development of present concepts of synaptic physiology. However, because of their inadequate oxygenation, these preparations might not have provided a good representation of the actual in vivo events. Creese et al. (1958) reported that the rate of oxygen consumption by rat diaphragm is about 0.027 cm³/cm²/min at body temperature. When one uses the standard oxygenation technique, Krebs solution simply cannot meet such a high demand for oxygen. (Creese, 1954; Creese et al., 1958; Calkins et al., 1954; Hubbard and Loyning, 1956, Paul, 1961). With the use of fluorocarbon emulsion it should be possible further to clarify the actual events which occur at the synapse.

In conclusion, the results of this study indicate that in vitro neuromuscular preparations bathed in fluorocarbon emulsion are superior to those bathed in Krebs solution. With the use of such emulsion, it will be possible to study drug effects and phenomena such as PTP which have not been readily observable in vitro.

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