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Mixed lubrication after rewetting of blotted pleural mesothelium

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Running head: PLEURA LUBRICATION AFTER REWETTING BLOTTED MESOTHELIUM

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Abstract

Coefficient of kinetic friction (μ) of pleural mesothelium blotted with filter paper, and rewetted with Ringer solution markedly increases; this increase is removed if a sufficient amount of sialomucin or hyaluronan is added to Ringer (Bodega et al., Respir. Physiol. Neurobiol. 180: 34-39, 2012). In this research we found that μ of pleural mesothelium blotted, rewetted, and sliding at physiological velocities and loads, decreased with increase of velocity, mainly at low velocities. Despite this decrease, μ at highest velocity was still double than before blotting. With small concentration of sialomucin or hyaluronan μ was markedly smaller at each velocity, decreased less with increase of velocity, and at highest velocity approached preblotting value. These finding indicate a regime of mixed lubrication in post-blotting Ringer, at variance with boundary lubrication occurring before blotting or postblotting with sufficient macromolecule addition. Greater roughness of mesothelial surface, caused by blotting, likely induces zones of elastohydrodynamic lubrication, which increase with velocity, while contact area decreases.

Keywords: Friction coefficient, Hyaluronan, Lubrication regime, Pleural mesothelium, Sialomicin, Sliding velocity

1. Introduction

D'Angelo et al. (2004) showed that the coefficient of kinetic friction (μ) between rabbit visceral and parietal pleura, during oscillatory sliding in vitro at physiological velocities and loads was 0.019 with pleural liquid between the mesothelia, and 0.027 ($P < 0.01$) with Ringer bicarbonate solution. These values did not change if the sliding velocity was increased from 0.9 to 3 cm/s: this is consistent with boundary lubrication. Under these conditions no damage of the sliding mesothelia was found by transmission electron microscopy. Moreover, they found that μ increased markedly after having blotted the mesothelial surface with filter paper for 1-2 min, and that this increase was reduced only a little by wetting the blotted mesothelium with Ringer solution. More recently, we showed that addition of sialomucin (25 mg/ml) or hyaluronan (2.5 mg/ml) in Ringer after a standard blotting of the mesothelium brought μ essentially back to its pre-blotting value, and that its value did not change when the sliding velocity was increased (Bodega et al., 2012). The same effect on blotted mesothelium was obtained by the addition of a mixture of sialomucin (12.5 mg/ml) and hyaluronan (1.25 mg/ml) in Ringer. Moreover, we found that, after washout of the solution with the macromolecules, μ with Ringer increased, without reaching its preceding post-blotting value. Given that the mesothelium is covered by a thick coat of polyanionic mucopolysaccharides (Andrews and Porter, 1973), mainly sialomucin (Wang, 1974 and 1985; Ohtsuka et al., 1997), our finding suggested that mesothelial blotting removes part of the macromolecules from the glycocalyx of the mesothelium, and their relevance for pleural lubrication. Finally, transmission electron micrographs of pleural specimens after mesothelial blotting showed that microvilli were partially or largely removed from the mesothelium, consistent with a substantial loss of the macromolecules normally entrapped among them (Bodega et al., 2012).

After blotting, therefore, the mesothelial surface is likely much more rough than it was before. Consequently, after the addition of Ringer the amount of liquid between the opposed mesothelial surfaces should be greater than that before blotting. It might, therefore, be that, when the visceral and parietal pleura are made to slide under physiological load, the shear induced hydrodynamic pressure,

besides reducing the roughness of the mesothelial surface (Gouldstone et al., 2003), generates some areas of elastohydrodynamic lubrication, which increase with the increase in sliding velocity. Hence, in post-blotting Ringer a regime of mixed lubrication might occur. If this were the case, μ in post-blotting Ringer should decrease with the increase in sliding velocity (see Loring et al., 2005), at variance with the constant μ at various velocities found in pre-blotting Ringer (D'Angelo et al., 2004, Bodega et al., 2012), and even after blotting if a sufficient concentration of sialomucin or hyaluronan is added (Bodega et al., 2012). Furthermore, because part of the boundary lubricant has been removed by blotting, the friction in the contact area should be markedly increased, but this component should decrease with the increase in sliding velocity, owing to the decrease in contact area. Finally, since contact area between the opposed mesothelia should still occur in post-blotting Ringer, a small concentration of the above mentioned macromolecules should cause a reduction in μ at each velocity. The purpose of the present research is, therefore, that of determining whether μ in post-blotting Ringer decreases with the increase in sliding velocity, and whether the addition of a small concentration of sialomucin or hyaluronan (i.e. insufficient to bring μ back to its pre-blotting value) decreases μ at each velocity.

2. Methods

Rib cage, lung, and diaphragm were obtained from 22 rabbits (2.6 – 3.5 kg b.w.) purchased from "G. Bettinardi", Momo (Novara). Animal experimentation was authorized by the Ministry of Health by decree N. 60/03A issued according to Order of the Excutive 116/92, in compliance with Directive 86/609/EC. Rabbits were anesthetized with an intravenous injection of 2 ml/kg of a mixture of pentobarbital sodium (Sigma, 12 mg/ml) and urethane (Sigma, 150 mg/ml). They were then heparinised (0.1 mg/kg) and killed by exsanguination. After removal of the skin and superficial muscles, the antero-lateral sides of the rib cage, the lungs (with closed trachea), and the diaphragm were removed. They were kept at ambient temperature (20 – 24 °C) in Ringer – bicarbonate solution (in mM: Na^+ 139, K^+ 5, Ca^{2+} 1.25, Mg^{2+} 0.75, Cl^- 119, HCO_3^- 29, D-glucose 5.6) through which 95% O_2 and 5% CO_2 was continuously bubbled. Up to 7 couples of specimen from each rabbit were used to measure the coefficient of kinetic friction (μ); no systematic difference occurred between the first and the last test, which was performed 3-4 h later.

The apparatus used to measure the frictional force was that described by D'Angelo et al. (2004). It consists of a sliding platform connected through unextensible threads to the core of a differential transformer (Lynearsyn Sanborn 565 DT), and a balance arm held stationary at its fulcrum by a force transducer. Tissues specimens to be tested were fixed to the sliding platform, which was driven sinusoidally over a distance of 1 cm by an electric motor, and to a plexiglas rod attached to one end of the balance arm, which could rotate to maintain contact between the sliding and stationary tissues. The balance arm was held horizontal, and counterweights added to its other end enabled to change the normal force applied to the tissue from ~ 0.5 to ~ 8 g. Because the cross section of the rod was 0.62 cm^2, the pressure acting on the mesothelium ranged from ~ 0.8 to ~ 12.9 cmH_2O. The frictional force on the direction of motion was measured by the force transducer. Under any given condition, the coefficient of kinetic friction (μ) was computed as the slope of the relationship between the frictional force and the load. The values of frictional force used were those recorded in the central 40% of the excursion, where a steady state velocity was achieved. This at variance with previous researches (D'angelo et al., 2004; Bodega et al., 2012) in which 80% of

the excursion was used. The specimen of the rib cage, with the pleural surface facing upwards, was fixed to the sliding platform by a peripheral frame that was screwed down. Alternatively, the specimen of the diaphragm, with the pleural surface facing upwards, was pinned to a flat cork on top of the platform. The specimen of the lung ~ 2 mm thick, with the pleural facing downwards, was held over the end of the rod with an O-ring. The experiments were made under the following sequence of conditions for each couple of specimens. 1) Ringer solution between specimens; 2) after blotting the mesothelium with filter paper (Bodega et al., 2012); 3) Ringer solution; 4) five min after having placed a solution with sialomucin (8 mg/ml; Sigma M3895) or hyaluronan (0.8 mg/ml; Sigma 53747); 5) Ringer solution after having washed out previous solution. Measurements performed with Ringer solution after mesothelial blotting and with macromolecule solution were performed at these sliding velocities: 0.9 cm/s, 1.9 cm/s, 2.8 cm/s, and 4.7 cm/s.

Linear regressions between frictional force and load were computed with the least squares method and statistical assessment was made by covariance analysis. The results are presented as mean ± S.E. Statistical significance of group mean values was assessed by analysis of variance. The level of significance was taken at $P < 0.05$.

3. **Results**

The coefficient of kinetic friction (μ) in post-blotting Ringer decreased markedly with the increase in sliding velocity as shown by Fig.1 (open circles). The decrease was greater between 0.9 and 1.9 cm/s, but was still significant ($P < 0.01$) between 2.8 and 4.7 cm/s. Despite the marked decrease in μ occurred with the increase in sliding velocity, μ in post-blotting Ringer at 4.7 cm/s (0.055 ± 0.003) was still markedly greater than in pre-blotting Ringer (0.026 ± 0.003, at 1.9 cm/s, Table 1, line 1), which is independent from sliding velocity (D'Angelo et al., 2004). After the addition of small concentration of sialomucin (8 mg/ml, Fig.1, open triangles), or hyaluronan (0.8 mg/ml, Fig.1, open squares), the values of μ at each velocity were markedly lower than those without the addition of these macromolecules. This difference was greater al low velocity, but was still ~ 30% ($P < 0.01$) at the highest velocity. The values of μ decreased with the increase in velocity, but to a smaller extent, and only with hyaluronan the decrease in μ between 2.8 and 4.7 cm/s was no longer significant. Moreover, these small concentrations of sialomucin or hyaluronan, that at low velocity are unable to bring μ close to its pre-blotting value, were able to do so at the highest velocity, though there was still a small significant difference. The values of μ at 4.7 cm/s were 0.038 ± 0.003 and 0.034 ± 0.002 for sialomucin and hyaluronan, respectively, while those in pre-blotting Ringer at 1.9 cm/s (which is independent from sliding velocity) were 0.024 ± 0.002 ($P < 0.01$), and 0.028 ± 0.002 ($P < 0.05$), respectively, as shown by Table 1 (line 2 and 3). In this Table (1st line) are also reported the μ values obtained in the same specimens as those in Fig.1 under different conditions and at a sliding velocity of 1.9 cm/s. These values are in line with those previously obtained (Bodega et al., 2012). Because the velocities used in our previous research were not 0.9 and 3 cm/s (as erroneously indicated in the paper, Bodega et al., 2012), but 1.9 and 4.7 cm/s, in the present research we measured μ at 0.9 cm/s in post-blotting Ringer with 2.5 mg/ml hyaluronan to provide further support to our previous finding that with this concentration μ does not change by changing the velocity. It was 0.030 ± 0.003 ($N = 10$). The values of μ in initial Ringer, blotting, post-blotting Ringer, and final Ringer in the same

specimens were 0.027 ± 0.002; 0.205 ± 0.023; 0.067 ± 0.009; 0.048 ± 0.05, respectively. They are in line with the corresponding values of our previous research (Bodega et al., 2012):

4. **Discussion**

The results show that the coefficient of kinetic friction (μ) in post-blotting Ringer decreases markedly with the increase in sliding velocity of the pleural mesothelial specimens (Fig. 1), but even at the highest velocity it does not come close to its pre-blotting value (Table 1), which is independent of sliding velocity (D'Angelo et al., 2004; Bodega et al., 2012). Moreover, the results show that with the addition of a small concentration of sialomucin or hyaluronan μ at each velocity is lower than in post-blotting Ringer, decreases with the increase in velocity (Fig. 1), and at the highest velocity becomes close to its pre-blotting value (Table 1). In this connection one may consider Stribeck curve in the version reported by Loring et al. (2005), which provides μ versus sliding velocity. According to this curve a decreasing μ with increasing sliding velocity indicates a regime of mixed lubrication (i.e. boundary and elastohydrodynamic). The marked decrease in μ with the increase in sliding velocity occurring in post-blotting Ringer is consistent with the smoothing of the rough surface of blotted mesothelium produced by the shear induced hydrodynamic pressure (Gouldstone et al., 2003; see Introduction). Hence, the elastohydrodynamic component of mixed lubrication increases with the increase in sliding velocity, while the boundary one decreases. Since in blotted mesothelium part of the boundary lubricant has been removed, the boundary component markedly contributes to the large value of μ at low sliding speed. This effect decreases with the increase in speed because the boundary component decreases, while the elastohydrodynamic one increases. The greater decrease in μ achieved at each velocity with the addition of a small concentration of sialomucin or hyaluronan in Ringer (relatively to Ringer alone, Fig. 1) is consistent with the simultaneous occurrence of boundary lubrication. This greater decrease in μ becomes smaller with the increase in sliding velocity (Fig. 1), because of the decrease in the friction contributed by the boundary component. On the whole, the findings obtained in post-blotting Ringer without and with a small concentration of the mentioned macromolecules are consistent with a regime of mixed lubrication. This is at variance with pre-blotting Ringer (see Introduction), when μ is low, and does not change with changes in sliding velocity, consistent with boundary lubrication (D'Angelo et al., 2004; Bodega et al., 2012). Indeed,

before blotting the mesothelial surface is rather smooth (see below), covered by macromolecules providing good boundary lubrication, and the amount of liquid between the opposed mesothelia is very small.

Fig. 2 provides a synoptic view of the decrease in μ at various velocities produced in post-blotting Ringer by the addition of sialomucin or hyaluronan at the concentration (25 and 2.5 mg/ml, respectively) sufficient to bring μ essentially back to its pre-blotting value (broken line, Bodega et al., 2012), and at 1/3 of these concentrations (dotted line, present research). For simplicity we first averaged at each velocity the similar values of μ obtained with sialomucin and hyaluronan; at the lowest velocity the value with the higher concentration refers to hyaluronan only, and has been obtained in this research (see Results). We then subtracted at each velocity the values obtained with the macromolecules from those obtained in post-blotting Ringer (Fig. 1, open circles). The values so obtained are plotted as closed (higher concentration) or open (lower concentration) diamonds in Fig. 2: hence, the interpolated broken and dotted lines provide the decrease in μ contributed through boundary lubrication by the higher and smaller concentration of the mentioned macromolecules, respectively. It appears that a greater concentration of these macromolecules is required to bring μ close to its pre-blotting values at slow velocities, when the fraction of contact areas should be relatively larger, as suggested by the regime of mixed lubrication occurring in post-blotting Ringer. It is as if the blotted mesothelium utilizes these macromolecules to restore the "slippery cushion" hypothesized by Andrews and Porter (1973) and the repulsive electric forces (Roberts, 1971). This does not imply that these macromolecules are those involved in boundary lubrication under physiological conditions (see below).

Recently, Kim et al. (2011) determined the stiffness and surface topography of specimens of rat parietal pleura by mean of the atomic force microscopy. The roughness normal to the surface was less than 10 μm (mostly ~ 5 μm). Considering the softness of the pleura, they concluded that under physiological conditions visceral and parietal pleura could conform to each other through the agency of local hydrodynamic pressure without requiring tissue-tissue contacts, i.e. elastohydrodynamic

lubrication should occur over the whole pleural surface. In discussing the view implying contact areas with boundary lubrication, these authors concluded that either the contact pressure should be so high as to damage the contacting tissues or the contact area should be much larger than suggested by microscopic observations. This point deserves a comment. The thickness of the pleural space has been measured in various species by reflex light microscope on quick frozen chest (Agostoni et al., 1968; 1969). The space appeared as a dark band between lung and chest wall because it does not reflect the light of the microscope; its width was similar in the superior and dependent regions, and increased markedly at bottom when a small volume of isotonic saline was injected into the space before freezing. When Andrews and Porter (1973) and Wang (1974) pointed out the relevance of microvilli and of the mucopolysaccharides entrapped among them for pleural mechanics it became apparent that they could provide contacts between visceral and parietal pleura (Murphy and Macklem, 1976; Agostoni, 1986). Indeed, because the microvilli with the enmeshed mucopolysaccharides should not reflect the light of the microscope (like the free cells of pleural liquid), and the length of the microvilli in the species examined is 2-3 μm, contacts between lung and chest wall should occur where the width of the dark band is ≤ 5μm. The histograms of the thickness of the pleural space obtained by reflex light microscopy (away from lobar margins) show that in dogs, cats, and rats ~ 40% of the measurements pooled around 5 μm. Hence, the width of the dark band was ≤ 5 μm in ~ 20% of the measurements, and the contact area between visceral and parietal pleura could be ~ 20% of the total. In rabbits and mice the width ≤ 5 μm occurred in ~ 15% and 25% of the measurements, respectively (Agostoni et al., 1968; 1969). A similar analysis cannot be made on the data of Lai-Fook and Kaplowitz (1985) because of the way in which pleural space thickness has been obtained. From Murphy and Macklem equation (1976), which provides the balance of forces within the pleural space, letting pleural surface pressure (Ppl) = – 3 cm H_2O, and pleural liquid pressure (Pliq) = – 5 cm H_2O, with a contact area of 20%, the contact pressure is 5 cm H_2O. At the end of a deep inspiration the difference between Pliq and Ppl increases, but the thickness of the pleural space decreases markedly (Agostoni et al., 1968), and, therefore, the percentage of contact area should increase. Consequently,

the contact pressure should not increase so much as to damage the mesothelium. Moreover, a very small contact area should occur through the cells of the pleural liquid (mainly monocytes and mesothelial cells) where the width of the space is 6-14 μm (Agostoni, 1972). Finally, the frequency distribution of the width of the pleural space is not symmetrical, most of the measurements being around 5 and 10 μm (Agostoni et al., 1968; 1969). This finding, as well as the similar width in superior and dependent regions, are consistent with the occurrence of contacts between visceral and parietal pleura (Agostoni, 1972). With regards to the difference between Ppl and Pliq one has to remind that this difference has also been found with the same device in the same place (Agostoni et al., 1989a). Measurements of the width of the pleural space by reflex light microscope in quick frozen specimens were also performed in sheep (Albertine et al., 1991). Though these authors concluded for no evidence of contact between visceral and parietal pleura, it appears from their histogram that the width was ≤ 11μm in at least 10% of the measurements. Given that the length of microvilli in sheep is 5-6μm (Albertine et al., 1982, Mariassy and Wheeldhom, 1983), the contact area through the microvilli could be ~ 10% of the total. The occurrence of contact areas does not prevent the continuity of pleural liquid. Lubrication should be boundary in the contact areas, and elastohydrodynamic elsewhere (Agostoni, 1972; 1986; Agostoni and D'Angelo, 1991; Agostoni and Zocchi, 2007). This view is compatible with small flows of pleural liquid caused by gravity, breathing and cardiac movements (Lai-Fook, et al., 1984; Agostoni et al., 1989b; Lai-Fook and Rodarte, 1991; Lai-Fook, 1998; 2004). Finally, one has to recall that despite the opposed recoil of the lung and the chest wall (that draw liquid into the pleural space) the volume of the pleural liquid under physiological conditions is kept to a minimum. This implies that there are mechanisms that absorb liquid from the pleural space and others that prevent a complete removal (von Neergaard, 1927; Agostoni, 1972). Evidences for mechanisms absorbing liquid from the pleural space have been provided (Agostoni 1972; Agostoni and Zocchi, 2007). Until there is no evidence for a negative chemical feedback preventing a complete liquid removal, it seems that the only mechanism is provided by the contact

between lung and chest wall (Agostoni, 1972; Agostoni and Zocchi, 2007) through suitable boundary lubricants (Andrews and Porter, 1973; Wang, 1974; Hills et al., 1992; Bodega et al, 2012).

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Legends

Fig. 1. Coefficient of kinetic friction (μ) versus sliding velocity during reciprocating movements of a specimen of visceral pleura against one of parietal pleura under conditions reported on inset. N is 15 for each open circle; 11 for each open triangle; 12 for open squares at 1.9 and 4.7 cm/s, and 8 for the remaining open squares. Vertical bars indicate SE.

Fig. 2. Decrease in coefficient of kinetic friction (Δμ) at various sliding velocities of pleural mesothelium produced in post-blotting Ringer by addition of high (broken line) or low (dotted line) concentration of sialomucin (25 and 8 mg/ml, respectively) or hyaluronan (2.5 and 0.8 mg/ml, respectively). Closed and open diamonds refer to mean of values obtained with each macromolecule, except for close diamond at slowest velocity which refers to hyaluronan only (see text).

Table 1

Coefficient of kinetic friction (μ) of pleural mesothelium sliding at 1.9 cm/s under conditions indicated below, which precede or follow those illustrated by Fig.1

Condition	Initial Ringer	Blotting	Post blotting Ringer	Final Ringer	N
○	0.026±0.003	0.179±0.019	–	–	15 (8 L–C; 7 L–D)*
Δ	0.024±0.002	0.200±0.017	0.083±0.007	0.071±0.005	11 (6 L–C; 5 L–D)
□	0.028±0.002	0.189±0.015	0.086±0.006	0.076±0.007	12 (8 L–C; 4 L–D)

Values are mean ± SE.

*L–C: lung–intercostal; L–D: lung – diaphragm.

Figure 1

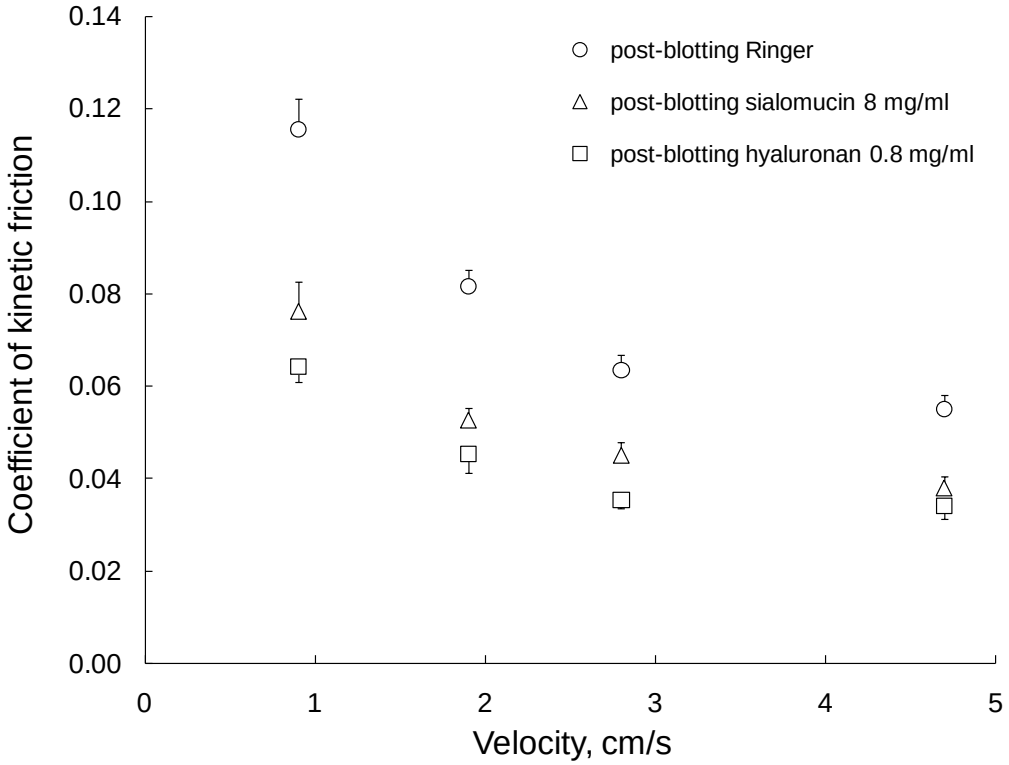

Fig. 1

Figure 2

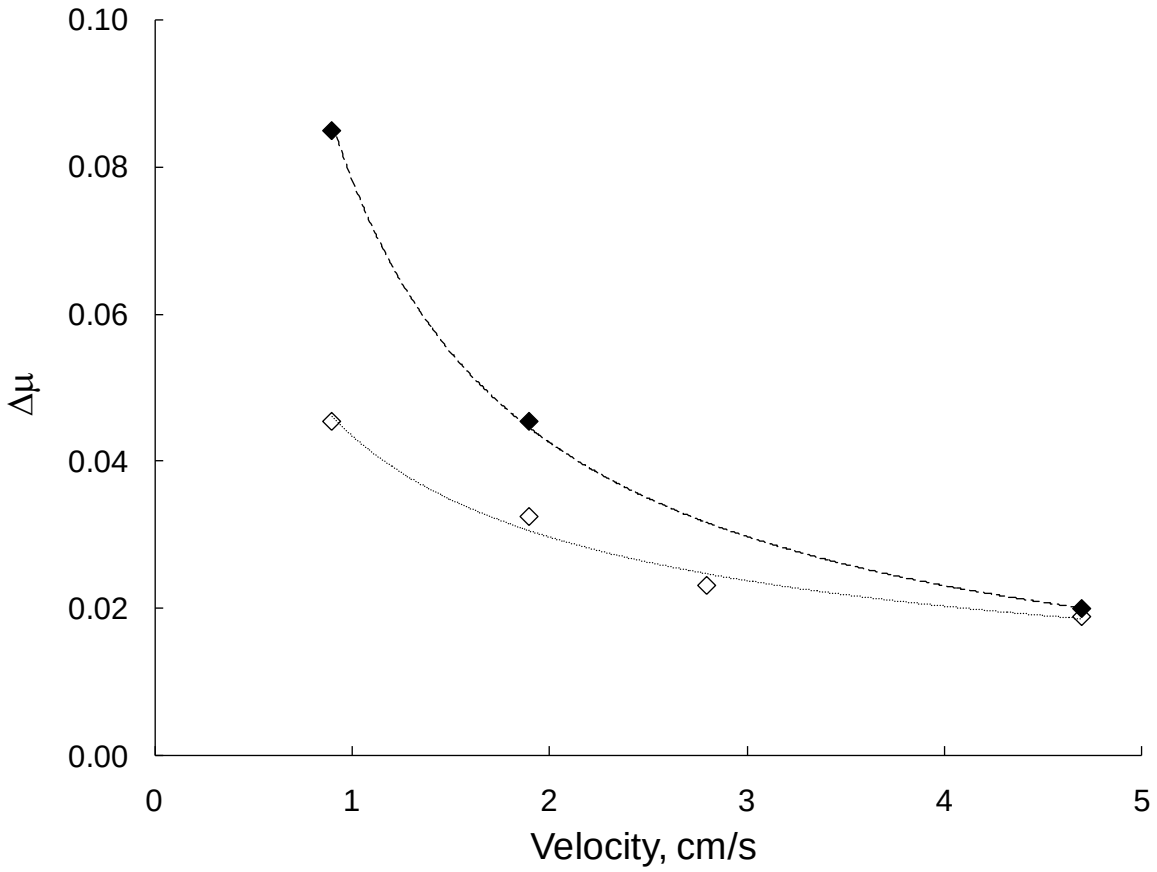

Fig. 2