In order to elucidate whether coffee brand differences account for the discrepancy between our results and those of Boublik et al.[1] we also investigated the capability of our instant coffee in antagonizing morphine in vitro. As in the paper of Boublik et al.[1] this was tested on the myenteric plexus-longitudinal muscle preparation from guinea pig ileum[7]. The muscle was set up isometrically in 36 °C tyrode and field-stimulated with rectangular pulses (0.1 Hz; 1 ms) of supramaximal voltage. After an equilibration period of 2–3 h, a dose-response relation was established by cumulative addition of morphine hydrochloride. The mean EC_{50} of morphine (n = 3) was 65 nM. Decaffeinated instant coffee (dissolved in bidestilled water) at a final concentration of 1 mg/ml shifted the concentration-response curve of morphine to the right, which resulted in a mean EC_{50} for morphine of 127 nM (n = 3). Additional experiments showed that this effect corresponded to that of 5 nM naloxone (= 2 ng/ml). Our instant coffee, therefore, also possesses the morphine-antagonistic in vitro activity demonstrated by Boublik et al.[1]. However, the efficacy of coffee in antagonizing morphine pharmacodynamically is very low indeed: One cup of coffee prepared according to manufacturer's recommendation from 2 g of instant coffee contains only the equivalent of one hundredth of an ampoule of naloxone. It must be stressed in this respect that the much higher estimation of Boublik et al.[1] according to which one cup of coffee corresponds to one-third of an ampoule of naloxone is based on binding studies and not on pharmacodynamic investigations.

In conclusion, coffee even in very high doses does not inhibit the pharmacodynamic activities of morphine in vivo. The in vitro results reported by Boublik et al.[1] and partially reproduced by us thus cannot be extrapolated to the whole organism in vivo. This is presumably due to the fact that the morphine-antagonistic activity of coffee is much too low as to be of any practical importance. There is no necessity, therefore, for patients needing the pharmacodynamic actions of morphine to avoid coffee consumption.

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Myelin changes in the rats CNS following intraventricular injection of serum[1]

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Summary. Normal human or rat serum administered by intraventricular injection induced demonstrable changes in the rat CNS myelin as seen from an increased recovery of dissociated myelin (DM), i.e. a myelin-related low density membrane fragments, from the tissue homogenates. The yield of DM reached a maximum on the third postinjection day and returned to the control level by day 5. In spite of the increased recovery of DM, no physico-chemical alternations in myelin isolates and no histological abnormalities in the tissue could be detected. The production of DM seems to be a sensitive index of serum-induced alteration of the myelin sheath.
Key words. Serum; intrathecal injection; CNS; dissociated myelin; demyelination; multiple sclerosis.

Abnormalities in the CNS blood vessels[3] followed by increased vascular permeability[4,5] may be among the earliest changes that procede demyelination in multiple sclerosis (MS). Some of the extravassating blood components may have a detrimental effect on the CNS tissue and contribute to the process of demyelination. We have recently demonstrated that the exposure of isolated myelin to serum results in gross chemical and physical changes in the membrane[6]. Similar myelin alterations can also be detected in cerebral slices following their incubation with serum[7]. The present experiments are a continuation of these studies aimed at establishing whether the myelin deteriorations can be induced in the CNS tissue exposed in vivo to intrathecally injected serum.

Material and methods. Adult male Wistar rats weighing about 300 g were anesthetized with Na-pentobarbital, 50 mg/kg. After fixation of the head in a stereotaxic device, a burr hole was made over the left parietal region and 100 µl (human or rat) serum or Krebs-Ringer bicarbonate buffer was injected intraventricularly using a Hamilton syringe with a 30G steel cannula. The coordinates from bregma were: 0 mm A/P, 2 mm L, 3.5 V[8].

Different anatomical parts of the CNS were dissected out and weighed. The tissue from two animals was combined and homogenized in 0.32 M sucrose. The material floating on 0.32 M sucrose (DM) and the myelin subfractions were isolated as previously described[7]. The electrophoretic analysis of membrane proteins was performed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate[9]. The immunoblot conditions were as described by Greenfield et al.[10].

Animals for histological examination were anesthetized with 1.5% Halothane and perfusion fixed with 300 ml Lillie's phosphate buffered formaldehyde through a cannula in the ascending aorta. The paraffin embedded brains were serially sectioned at 7 µm and stained with cresyl violet and luxol fast blue.

Results and discussion. No mortality or changes in the normal behavior of rats were observed following the intraventricular injection of human (or rat) serum or Krebs-Ringer bicarbonate buffer. The examination of the brain tissue at the light microscopic level revealed no large hemorrhages, cellular infiltrations or infarcts. Around the cannula track in the cortex a few macrophages with hemosiderin granules and some necrotic nerve cells were seen. Systematic examination of myelin stained specimens showed some myelin loss around the cannula track in corpus callosum with a few luxol fast blue positive swollen macrophages. There was also vacuolization of myelin sheaths in the surroundings of the tract. These changes seemed to be more pronounced in the serum injected animals. No remote signs of focal or diffuse demyelination were found. Our findings corroborate those from other laboratories reporting no pathological changes in the tissue due to intrathecal injection of control serum[11,12].

The physico-chemical changes in myelin observed in the system of tissue slices following incubation with serum, i.e. the increase

in buoyant density of myelin particles, the loss of basic protein and the absorption of serum proteins[7], could not be traced in the present in vivo system. The only common serum-induced alteration detected in both systems was increased recovery of a material floating on 0.32 M sucrose. This low density fraction frequently referred to as 'dissociated myelin' or 'floating fraction' has been found elevated in the CNS tissue of animals with experimentally induced demyelination[13–16] and is generally believed to represent a degradation product of myelin sheath undergoing vesicular type disruption[13].

The relationship between dissociated myelin (DM) and myelin fraction becomes conspicious upon comparison of the protein moieties of these membranes. DM contains typical myelin proteins as seen from the band pattern, however it is significantly enriched in high molecular weight proteins (fig. 1A). Immunoblot analysis (fig. 1B and C) provides further evidence for the identity of bands in DM with the major myelin proteins, i.e. proteolipid protein (PLP), intermediate protein (IP) (a species cross-reacting with PLP), and basic proteins (BP).

The yield of DM isolated from the tissue depended on the post injection time, however, no abnormalities in the protein composition of this material were detected at any time point. The results are delineated in figure 2. A certain amount of DM was normally isolated from CNS tissue and no changes in the yield were detected following the injection of Krebs-Ringer bicarbonate buffer. The amount of DM increased after the treatment with human serum and reached a maximum on day 3. At that time the elevation over the control value was 180% and 242% for forebrain and brain stem respectively. The difference between the control and serum-injected animals as calculated by Students's t-test was strongly significant for both tissues ($p \leqslant 0.001$). The amount of DM decreased to the control level on day 5. Also rat serum possessed the DM-generating activity. The time-course of DM production induced by rat serum was essentially identical to that elicited by human serum. The increase in the amount of DM found on the third postinjection day was similar to the increase observed in animals injected with human serum and was 170% and 231% in the case of forebrain and brain stem respectively ($p \leqslant 0.001$ for both tissues). Thus, serum as such seems to contain the activity regardless of its generic origin.

The response of forebrain tissue appears to be faster than the response of brain stem tissue; one day after the injection of serum, increased amounts of DM could be detected in forebrain but not in brain stem. The response of the brain stem was, however, more pronounced as seen from the maximal increase in DM. The dissimilarity of timing and extent of response between the tissues was probably due to combined effect of factors such as injection site and tissue susceptibility[7]. The serum-induced elevation of DM was also detected in the spinal cord (result not shown). The average increase on day third, calculated from two independent determinations was found to be about 25%.

The extent of serum-induced elevation over the control value in the yield of recoverable DM in the in vivo system was smaller when compared to the slice system[7]. As compared to the tissue slice technique the in vivo system allows studies over significantly extended periods of time and the effect of necrotic changes concurrent with the incubation in vitro is eliminated. Furthermore, the in vivo system provides better approximation to the conditions following an in situ extravassation. On the other hand, the active concentration of serum cannot be assessed due to its dilution with the cerebrospinal and interstitial fluids and due to the physiological flow of these fluids. Factors such as tissue integrity and the active concentration of serum most likely contributed to the observed quantitative dissimilarities between the two systems.

The mechanisms by which DM particles dissociate from the myelin sheath in vivo are not elucidated. The particles may represent artifacts of homogenization; nevertheless, their increased recovery from the affected tissue reflects some subtle changes in the myelin sheath in vivo. Thus, one may consider the possibility that serum only initiates the process by causing a segregation of membraneous material in some of the myelin sheath domains and that these altered low density parts are subsequently sheared off during the homogenization process to yield DM. The transient accumulation of the DM in the course of the experiment suggests reversibility of the serum-induced myelin sheath damage.

The subtle myelin alterations may become more salient upon prolonged exposure to serum as could be expected during an in situ extravassation. Such a situation can be envisaged to occur in

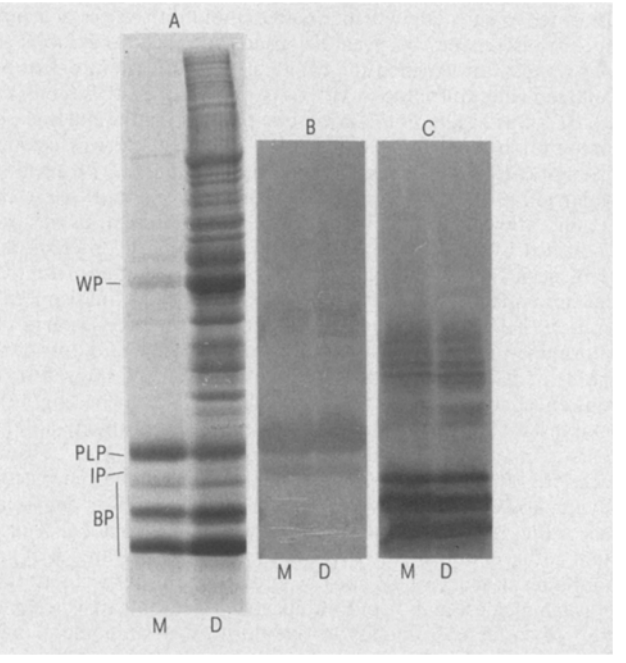

Figure 1. Protein composition of rat cerebral myelin (M) and dissociated myelin (D). *A* Protein pattern on 10–25% polyacrylamide gradient gel stained with Coomassie brilliant blue; *B* Immunoblot stained with rabbit anti-PLP antiserum; *C* Immunoblot stained with rabbit anti-BP antiserum. Myelin proteins are designated as follows: WP, Wolfgram protein; PLP, proteolipid protein; IP, intermediate protein; BP, basic proteins.

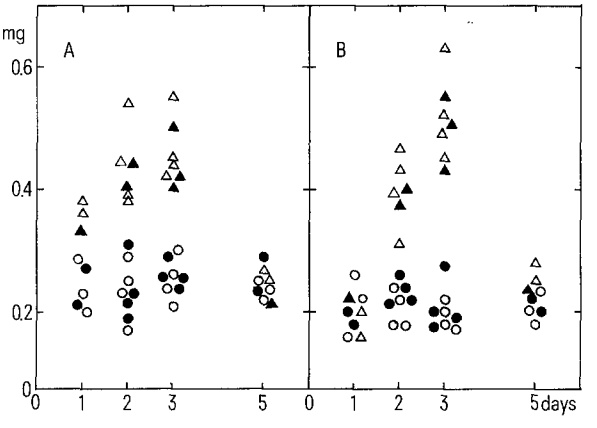

Figure 2. The amount of dissociated myelin in the rat forebrain (A) and brain stem (B) tissue following intraventricular injection of serum. The results are expressed as milligrams of dry weight of DM per gram of fresh tissue. The points represent values from separate experiments. Two animals were used in each experiment. ○, control (uninjected); ●, Krebs-Ringer bicarbonate buffer-injected; △, human serum-injected; ▲, rat serum-injected.

MS, a demyelinating disease in which the impairment of blood-brain barrier is well documented[4,5]. At the present stage it cannot be argued that the serum factors responsible for the generation of DM ultimately cause myelin disintegration, however they may contribute to the process by rendering the sheath more susceptible to other humoral and cell-mediated demyelinating agents. The above in conjunction with augmented myelinolytic activity of sera from MS patients[17-19] is likely to contribute to the pathogenesis of this disease.

In conclusion, the present study demonstrates that the CNS myelin in vivo is vulnerable to some components present in normal serum. By as yet unknown mechanisms these factors induce membrane changes in the sheath leading to the generation of low density fragments (DM). The increased recovery of DM from the tissue homogenates seems to be an early and sensitive index of serum-induced myelin alterations.

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Trypsin in human milk

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Summary. Human milk trypsin was purified by adsorption chromatography on cellulose-bound 4-aminobenzamidine; its molecular weight was about 24,000 daltons. Its concentration determined by a radioimmunoassay varies between 2.9 and 5.6 µg/l.
Key words. Human milk; trypsin; affinity chromatography; HPLC; radioimmunoassay.

Human milk contains a larger amount of proteolytic enzymes than cow milk[1] but the identity of the proteases is not yet completely established. Only Borulf et al. reported the isolation of a trypsin-like enzyme from human colostrum by immunoadsorption chromatography[2]. In the present note we report the trypsin content of human milk determined by a radioimmunoassay and the purification of the enzyme from human colostrum and milk by adsorption chromatography on cellulose-bound 4-aminobenzamidine, followed by high pressure liquid chromatography on a TSK-SW-type column.

Materials and methods. Human milk samples from healthy mothers were obtained at the hospital and from individual donors. Milks were defatted by centrifugation and frozen until use. Radioimmunoassay was performed according to Temler and Felber[3] with trypsin-specific immunochemicals from Serono Diagnostics (Coinsins, Switzerland): the antibodies did not cross-react with chymotrypsin (EC 3.4.21.1), thrombin (EC 3.4.21.5), plasmin (EC 3.4.21.7) and elastase (EC 3.4.21.11). Affinity chromatography on cellulose-bound 4-aminobenzamidine was performed as indicated elsewhere[4]. 0.5 ml of moist adsorbent was mixed with 100 ml milk and gently stirred for 3 h at room temperature. The desorbed material was dialyzed against 10 mM HCl. Active trypsin was determined by the procedure elsewhere described[4]. To detect whether the enzyme was active in milk, 1 ml of 10 mM BAPA (benzoyl-L-arginine p-nitroanalide) reagent was added to 10 ml of a milk sample. After incubation for 12 h at 37°C, 200 µl of 24% trichloroacetic acid were added and the precipitate removed by centrifugation (20,000 g; 30 min). The supernatant was filtered through Millipore 0.22 µm filters (Millex-HC, Millipore, Bedford) and measured at 410 nm.

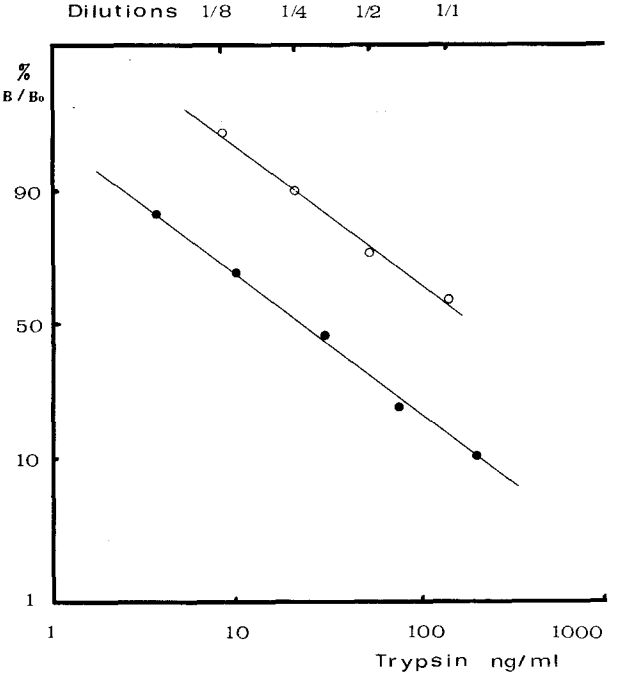

Figure 1. Logit plot of the reference calibration (curve) (●) and serial dilution of a pool of milks (○). B_0, radioactivity in absence of unlabeled antigen. B, radioactivity of the bound fraction.