



Quantitation of major protein constituents of murine intestinal fluid

Steffen Bade^a, Hans H. Gorris^{a,1}, Sabine Koelling^b, Verena Olivier^{a,2}, Fabian Reuter^a, Peter Zabel^c, Andreas Frey^{a,*}

^a Division of Mucosal Immunology, Research Center Borstel, 23845 Borstel, Germany

^b Central Chemical Analytical Laboratory, Technical University, 21073 Hamburg, Germany

^c Department of Pneumology, Research Center Borstel, 23845 Borstel, Germany

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ABSTRACT

The gastrointestinal tract is a hostile biological environment, yet not all ingested materials are destroyed. The minute differences that determine whether a substance persists or is digested, liberated, adsorbed, excreted, or taken up are still poorly understood. Most attempts to investigate the events occurring during an orogastrintestinal passage rely on simplified in vitro systems where an analyte is exposed to artificial intestinal fluids. To closely mimic the events in the gastrointestinal tract, the exact intestinal fluid composition and the in vivo concentration of its constituents must be known. The widely used lavage procedures, however, dilute the intestinal fluids to an extent that precludes recalculation to the original concentrations. Thus, we developed procedures with which undiluted murine intestinal fluid can be harvested; determined the in vivo concentrations of the digestive enzymes trypsin, chymotrypsin, and elastase and the adsorbents mucin and immunoglobulin A in small intestinal fluid of fasted and unfasted female Balb/c mice; and identified chymotrypsin and immunoglobulin A as valid endogenous dilution markers for the recalculation of aqueous lavages. With these technologies and information at hand, more reliable investigations on the fate of allergens, pathogens, food, and anthropogenic xenobiotics in the gastrointestinal tract will be possible.

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The orogastrintestinal tract represents a harsh environment to any foreign substance. Ingested materials are attacked by digestive enzymes, absorbing mucus, and the defensive machinery of the mucosal immune system. Most food constituents, pathogens, and noxae do not survive these physical, chemical, and biological challenges and are broken down or otherwise eliminated. Hence, drug and vaccine developers constantly face the challenge to protect their agents against premature destruction. On the other hand, certain food proteins and a number of pathogens are able to survive the gastrointestinal environment [1,2] and, thus, may give rise to allergies and infectious diseases. The specific properties and/or conditions that render materials resistant or susceptible to gastrointestinal destruction are poorly understood.

So far, we know that the presence and amount of bystander protein may determine whether proteinaceous vaccine delivery systems are digested or not [3]. Digestion-resistant pathogens and

food allergens, although not broken down, still may be eliminated by the mucosal immune system if it is triggered properly [4,5]. The fate of many anthropogenic synthetic substances such as artificial nanoparticles is even less clear. Many of these compounds are neither immunogenic nor digestible. Whether the gastrointestinal tract ignores such structures, or whether they are attacked by other means or taken up, remains to be elucidated [6,7].

To investigate these problems in more detail, it is important to mimic the in vivo situation in the gut as closely as possible. This in turn requires the availability of undiluted intestinal juices or information on the actual in vivo concentrations of the major fluid components. Although harvesting diluted intestinal fluid is simple, it is far more challenging to obtain such fluids in undiluted form or to determine the degree of dilution in intestinal flushings. We established and compared different procedures to harvest undiluted intestinal fluid and quantitated the in vivo concentrations of prominent intestinal solutes therein. In addition, we investigated whether aqueous lavages yield representative fractions of intestinal fluid and identified endogenous dilution markers that are suitable to determine the dilution of intestinal fluid in a lavage preparation and to extrapolate to the in vivo situation.

* Corresponding author. Fax: +49 4537 188 693.

E-mail address: afrey@fz-borstel.de (A. Frey).

¹ Present address: Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, 93040 Regensburg, Germany.

² Present address: Intercell, Campus Vienna Biocenter 3, 1030 Vienna, Austria.

Materials and methods

Animals

Female Balb/c mice ($n = 40$, 11 weeks old) were obtained from Harlan Winkelmann (Borchen, Germany) and kept on a rodent chow diet (ssniff R/M-H, Soest, Germany). The mice were divided into two groups of 20 animals each (groups I and II). Half of the animals of each group were fasted for 2.5 h (water ad libitum) before all of them were anesthetized by short isoflurane inhalation and killed by cervical dislocation for immediate harvesting of the intestinal secretions.

Materials and reagents

UniWick filters (25 mm length, 2.5 mm diameter) were obtained from Polyfiltronics (Rockland, MA, USA), and glass applicator tubes (10 cm length, 4.0 mm o.d., 2.5 mm i.d., smoothed and beveled at one end) were custom-made by Glasgerätebau Ochs (Bovenden-Lengler, Germany). Polystyrene microtiter plates (high-binding or nonbinding) were obtained from Corning Costar (Bodenheim, Germany).

Isoflurane was obtained from Baxter (Unterschleißheim, Germany). Perfluorohexane (FC-72), perfluorotributylamine (FC-43), and perfluorotripentylamine (FC-70) were provided by 3 M (Neuss, Germany). The cesium ion (Cs^+)³ standard (CsNO_3 in HNO_3) and the lithium ion (Li^+) standard (LiNO_3 in H_2O) were obtained from Merck (Darmstadt, Germany), and the rhodium (Rh) standard (RhCl_3 in HCl) was obtained from Kraft (Neumühl, Germany).

Leupeptin hydrogen sulfate and 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) hydrochloride were from Merck Biosciences (Bad Soden, Germany). Aprotinin, bestatin hydrochloride, trypsin (bovine, EC 3.4.21.4, 12,400 BAEE U/mg protein), chymotrypsin (bovine, EC 3.4.21.1, 67 BTEE U/mg protein), and pancreatic elastase (porcine, EC 3.4.21.36, 34 BAEE U/mg protein) were purchased from Sigma-Aldrich (Taufkirchen, Germany), and Bz-Arg-pNA, Suc-Ala-Ala-Pro-Ala-pNA, and Suc-Ala-Ala-Pro-Phe-pNA were purchased from Bachem (Weil am Rhein, Germany).

Peroxidase-labeled, heavy-chain-specific goat anti-mouse immunoglobulin A (IgA), unlabeled heavy-chain-specific goat anti-mouse IgA, and purified mouse IgA were obtained from Southern Biotechnology Associates (Birmingham, AL, USA, via Biozol, Eching, Germany), and Cy5-labeled goat anti-rabbit immunoglobulin G (IgG) was purchased from Zymed (San Francisco, CA, USA). The polyclonal rabbit anti-mucin antibody was kindly provided by J. Forstner (University of Toronto and Hospital for Sick Children, Toronto, Canada).

Sample collection and extraction

Intestinal fluids were sampled from small intestines of mice using either a three-tiered lavage technique or a direct harvesting method with filter wicks for fluid absorption similar to the procedure for immunoglobulins described by Haneberg and coworkers [8]. The aqueous SIF (simulated intestinal fluid) buffer (1.64 mM KH_2PO_4 , 2.97 mM KCl, 6.10 mM Na_2HPO_4 , and 99.40 mM NaCl) used for the lavage was adapted to the natural electrolyte concen-

trations according to Lockwood and Randall [9] and was optionally spiked with tracer ions (SIF*: 1.64 mM KH_2PO_4 , 2.97 mM KCl, 6.10 mM Na_2HPO_4 , 97.95 mM NaCl, 1.15 mM LiCl, and 0.30 mM CsCl). The animals were killed, their small intestines were dissected carefully, and intestinal secretions were sampled immediately by either the lavage technique (group I) or the filter wick method (group II).

Three-tiered lavage technique

A syringe with a blunt needle was used to drive different solutions sequentially from proximal to distal through a dissected small intestine. First, the lumen of the intestine was flushed twice with 6 ml of perfluorohexane and 4 ml of air (perfluorohexane expulsion) and then with a mixture of 2.25 ml of perfluorohexane, 0.25 ml of tracered SIF* buffer, and 7.5 ml of air (tracered wash). The remaining luminal contents were eluted with 5 ml of SIF buffer (untracered wash). The three fractions were centrifuged at 4000 and 16,000g at 4 °C, each for 3 min, and the aqueous phases were separated from perfluorohexane and feces. Samples intended for determination of mucin or IgA content were furnished with protease inhibitors (final concentration: 154 nM aprotinin, 1 μM leupeptin, 200 μM AEBSF, 6 μM bestatin, and 1 mM ethylenediaminetetraacetic acid [EDTA]). All samples were snap-frozen in liquid nitrogen and kept at -80°C .

Filter wick method

The dissected intestine was carefully slipped over a glass applicator tube, and the luminal contents—secretions and solids—were collected in the tube and absorbed into two preweighed filter wicks (applicator collection). Nine preweighed filter wicks were then inserted into the intestine, with three of them covering each of the proximal, medial, and distal parts, and left in place for 5–10 min on an ice-cold plate to absorb the local secretions (absorption wicks). All wicks were checked visually for the absence of blood, weighed, snap-frozen in liquid nitrogen, and stored at -80°C . Extraction of filter wicks was performed as described previously [8].

The filter wicks were weighed, and the mass of the absorbed material was determined. Ice-cold extraction buffer (Dulbecco's phosphate-buffered saline [D-PBS: 2.7 mM KCl, 1.5 mM KH_2PO_4 , 136 mM NaCl, and 8.1 mM Na_2HPO_4 , pH 7.3] containing 5% [w/v] nonfat dry milk, 6 μl [applicator collection wicks] or 30 μl [absorption wicks] per milligram absorbed material) was added, and the filter wicks were incubated at 0 °C for 30 min with occasional mixing. The buffer was collected, and all remaining liquid was harvested from the soaked filter wicks by 2 min centrifugation at 16,000g. The extracts were cleared by 4 min of centrifugation at 16,000g. Solid matter (feces) was removed and lyophilized, and the dry weight was subtracted from the previously determined mass of absorbed material to determine the true amount of secreted juices. A protease inhibitor cocktail was added to the samples intended for the determination of mucin or IgA as described above. The filter extracts were snap-frozen in liquid nitrogen and stored at -80°C .

Histological analysis

From each intestine treated with the three-tiered lavage technique as well as from five untreated intestines, 2.5-cm-long tissue samples from the medial part were fixed for 2.5 h in 4% (v/v) formaldehyde, dehydrated with an ascending ethanol series and xylene, and finally embedded in 100% (w/v) ParaPlast Plus (Sherwood Medical, St. Louis, MO, USA). Serial sections (2 μm) were captured on glass slides (Menzel-Gläser, Braunschweig, Germany) and dyed automatically with periodic acid Schiff (PAS) stain in a tissue stainer (TST 40, Medite, Burgdorf, Germany). For comparison, five non-treated intestines were prepared analogously.

³ Abbreviations used: Cs^+ , cesium ion; Li^+ , lithium ion; Rh, rhodium; AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride; IgA, immunoglobulin A; IgG, immunoglobulin G; SIF, simulated intestinal fluid; EDTA, ethylenediaminetetraacetic acid; D-PBS, Dulbecco's phosphate-buffered saline; PAS, periodic acid Schiff; ICP-MS, inductively coupled plasma-mass spectrometry; ICP-OES, inductively coupled plasma-optical emission spectroscopy; L-BAPA, *N*- α -benzoyl-Arg *p*-nitroanilide; Suc-AAPF-pNA, succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide; Suc-AAPA-pNA, succinyl-Ala-Ala-Pro-Ala *p*-nitroanilide; PBST, D-PBS containing Tween 20; RT, room temperature; PBS-Blotto, D-PBS containing nonfat dry milk; ANOVA, analysis of variance.

Determination of ion concentrations

All samples intended for determination of their tracer ion content were diluted with H₂O (Li⁺, 100-fold; Cs⁺, 5000-fold). A ¹⁰³Rh solution was added (final concentration: 10 ng/ml) for internal standardization.

The isotope ¹³³Cs⁺ was measured by inductively coupled plasma–mass spectrometry (ICP–MS) with a SCIEX ELAN 6000 (Perkin-Elmer, Fremont, CA, USA). The instrument was calibrated with a Cs⁺ standard (40, 20, 10, 5, 2, and 1 ng/ml, $R^2 = 0.99$, detection limit = 0.5 ng/ml). Instrument settings were 15 L/min plasma gas, 0.5 L/min auxiliary gas, 0.9 L/min nebulizer gas, and RF power 1000 W, with a sample uptake rate of approximately 1.0 ml/min.

Li⁺ was analyzed by inductively coupled plasma–optical emission spectroscopy (ICP–OES) with an Optima 2000 DV OES with ICP (PerkinElmer). Calibration was done with a Li⁺ standard (100, 500, 200, 100, 50, and 20 ng/ml, $R^2 = 0.99$, detection limit = 35 ng/ml), as an internal standard Y (1 µg/ml, 371 nm) was used. The samples were measured at 670 nm (Li). Instrument settings were 15 L/min plasma gas, 0.4 L/min auxiliary gas, 0.75 L/min nebulizer gas, and RF power 1300 W, with a sample uptake rate of approximately 1.5 ml/min.

Determination of total protein and enzymatic concentrations

The total protein content of the intestinal fluid was determined with the DC Protein Assay (Bio-Rad, München, Germany).

Trypsin-, chymotrypsin-, and pancreatic elastase-like activities were determined with the colorogenic substrates *N*- α -benzoyl-Arg *p*-nitroanilide (α -BAPA), succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-pNA), and succinyl-Ala-Ala-Pro-Ala *p*-nitroanilide (Suc-AAPA-pNA), respectively. The substrate stock solutions (50 mM α -BAPA in acetone/H₂O [50:50] and 10 mM Suc-AAPF-pNA or Suc-AAPA-pNA in acetone) were diluted with SIF containing 0.1% (v/v) Tween 20 to a final concentration of 1.3 mM. Diluted substrate solution (75 µl) was distributed in the wells of a non-binding microtiter plate, and intestinal fluid or the individual proteases were added and serially diluted for a dataset of four different concentrations. A microplate reader (Molecular Devices, Wokingham, UK) was equilibrated to 37 °C, and substrate hydrolysis was measured at 405 nm for 2 h in 2-min intervals. Enzyme activities were calculated according to Eq. (1):

$$\text{Units}(\mu\text{mol}/\text{min}) = \frac{F_D \times V_{\max}}{\varepsilon_{\text{pNA}} \cdot l}, \quad (1)$$

where $V_{\max}$ ($\Delta\text{OD}/\Delta t$ [min]) is the linear portion of the data curve (10 continuous time points), ε_{pNA} is the extinction coefficient of *p*-nitroaniline at 405 nm ($7958 \text{ mol}^{-1} \text{ cm}^{-1}$), l is the light path (0.213 cm), and F_D is the dilution factor. The enzyme activities were converted to concentrations (mg/ml) by comparison with the activity of standard enzyme solutions.

Quantitation of total IgA

For determination of the total IgA content, high-binding microtiter plates were coated with 75 µl per well of 5 ng/ml unlabeled goat anti-mouse IgA in D-PBS overnight at 4 °C. The plates were washed three times with 300 µl of D-PBS containing 0.05% (v/v) Tween 20 (PBST), blocked for 5 h at room temperature (RT) with D-PBS containing 5% (w/v) nonfat dry milk (PBS–Blotto), and washed again before 75 µl of serially diluted IgA standard (purified mouse IgA, starting concentration 16 mg/ml), washes, or filter extracts in PBS–Blotto were applied and incubated overnight at 4 °C. After washing, captured IgAs were detected with horseradish peroxidase-labeled goat anti-mouse IgA (1 mg/ml, 1:2000 in PBS–

Blotto, 90 min, RT), excess detection reagent was washed off, and color was developed with tetramethylbenzidine substrate reagent containing 1 mM 3,3',5,5'-tetramethylbenzidine and 3 mM H₂O₂ in 200 mM potassium citrate buffer (pH 4.0) [10]. The color reaction was terminated after 30 min by the addition of 125 µl of 1 M sulfuric acid, and the plates were read at 450 nm on a microplate reader. Total IgA amounts were determined on the basis of four-parameter curve fit approximations of IgA standard titration curves using the readouts of the unknown samples at the steepest slopes of their titration curves (Softmax Pro, version 4.7, Molecular Devices, Ismaning, Germany).

Analysis of mucin content

Mucin concentrations in lavage or filter extracts were determined by dot blot analysis. Mucin purified from mouse intestine via two CsCl gradients (purification procedure modified from Ref. [11]) served as a standard, and 1.5 µl of each sample was immobilized on a nitrocellulose membrane. The air-dried membrane was washed three times for 5–10 min in D-PBS and twice in PBST and subsequently blocked for 1 h at RT with PBS–Blotto and incubated overnight at 4 °C with rabbit anti-mucin serum (1:5000 in PBS–Blotto). Before and after the incubation with the secondary antibody (goat anti-rabbit IgG–Cy5 conjugate, 1 mg/ml, 1:2000 in PBS–Blotto, 90 min, RT), the membrane was washed five times for 10 min with PBST and two times with D-PBS. The fluorescence signals were quantified using an Odyssey Infrared Imaging System and the Odyssey software (version 1.2.15) (LI-COR Biosciences, Bad Homburg, Germany).

Data analysis and statistics

The volume of undiluted intestinal fluid [$V(\text{IF})$], harvested either by displacement with perfluorohexane (perfluorohexane-expelled fraction of the three-tiered lavage technique) or by elution from filter wicks, was determined by weight (1 mg = 1 µl). For the aqueous wash obtained by flushing with tracer ion-spiked buffer (tracered wash of the three-tiered lavage technique), the corresponding $V(\text{IF})$ was approximated via the exogenous markers of dilution. The sample dilution factor (DF) was calculated from the concentrations of the tracer ions, Li⁺ and Cs⁺, in SIF⁺ (c_0) and in the tracered wash of the lavage ($c_{\text{tracered wash}}$) using Eq. (2):

$$DF = \left(1 - \frac{c_{\text{tracered wash}}}{c_0}\right)^{-1}. \quad (2)$$

The respective volume of undiluted intestinal fluid in the tracered wash [$V(\text{IF})_{\text{tracered wash}}$] was calculated by dividing the total volume of the tracered wash by the sample dilution factor DF .

Results are presented as means and ranges (lower–upper 95% confidence intervals of the mean) of 6–10 animals. Statistical analyses were carried out with GraphPad Prism (version 5.01, GraphPad Software, San Diego, CA, USA). Between-group comparisons were performed by one-way analysis of variance (ANOVA) with Bonferroni post hoc test. One-sample *t* tests were used to decide whether a group mean was different from 100%. For all statistical analyses, a probability of $P < 0.05$ was considered as significant.

Results

To establish a valid in vitro assay for predicting the behavior of ingested substances, we determined the in vivo concentrations and distributions of small intestinal solutes such as digestive enzymes and adsorbents.

Isolation of undiluted murine small intestinal fluid

In a first attempt to harvest undiluted small intestinal fluid, we tested whether it can be squeezed out mechanically. This turned out to be not practicable because we could not rule out that the physical force applied during this treatment damages the intestinal tissue and, thus, may alter the composition of the isolated intestinal fluid (data not shown). Therefore, alternative procedures to isolate intestinal juice were established (Fig. 1).

We first evaluated three water-immiscible and chemically inert liquids—perfluorotributylamine, perfluorotripentylamine, and perfluorohexane—for use as a soft displacement tool for the aqueous intestinal fluid. Only perfluorohexane, which has the lowest kinematic viscosity (0.4 cSt) of the liquids tested, was suitable to expel undiluted intestinal juice. The perfluorohexane-displaced fluid gained by this procedure (Fig. 1A) had a mean volume of 21 μ l (range = 3–39 μ l). To ensure that perfluorohexane did not influence the proteolytic activities of digestive enzymes, we conducted pro-

tease activity tests with pNA substrates specific for prominent enzymes in intestinal juice, namely trypsin, chymotrypsin, and pancreatic elastase. Pure enzyme solutions or an aqueous intestinal lavage was intensely mixed with perfluorohexane and, after phase separation, the enzyme activities in the respective aqueous phases were determined. Perfluorohexane did not alter the enzymes' activity as compared with enzyme preparations that had not been treated with perfluorohexane (data not shown). To analyze whether the nutritional state of the animals (i.e., the presence of food pulp in the small intestine) influences the extraction of intestinal juices, one group of animals was deprived of food for 2.5 h before the small intestine was dissected. The volume of intestinal juice isolated by perfluorohexane expulsion from fasted animals was 14 μ l (range = 0–29 μ l) and did not differ from the amount obtained from unfasted animals (unpaired two-tailed *t* test, *P* = 0.517).

To determine whether perfluorohexane displacement yielded the entire murine intestinal juice, we compared this method with an isolation procedure where we modified a technique that had been developed by Haneberg and coworkers [8] for the determination of IgA at the intestinal mucosa after mucosal immunization. In our harvesting method (Fig. 1D and E), the intestine is slipped over a glass applicator, which skims off and collects the intestinal contents in its inside. An additional fraction of intestinal juice is subsequently absorbed from the gut walls by filter wicks that are placed in the intestine itself. By applying this technique, we were able to isolate 196 μ l (range = 135–257 μ l) of luminal intestinal juice from fasted animals and 147 μ l (range = 96–198 μ l) from unfasted animals with the glass applicator. Extraction of the intestinal wall wicks yielded another 66 μ l (range = 55–78 μ l) and 91 μ l (range = 80–101 μ l) of fluid, respectively.

The volume of intestinal juice obtained with the glass applicator exceeded that of the one isolated by perfluorohexane displacement by a factor of more than 10. Perfluorohexane obviously effectuated the isolation of only a very mobile luminal fraction of the intestinal fluid; most of the luminal content could not be displaced in this way. Therefore, we tested whether the remainder can be mobilized by a subsequent aqueous lavage.

Isolation of diluted small intestinal fluid by aqueous lavage

Aqueous lavages are widely used to harvest intestinal contents. An unavoidable drawback of aqueous lavages, however, is the sample dilution. To determine the dilution of intestinal fluid in such an aqueous lavage, we spiked the lavage buffer with nonphysiological alkali ions, Cs⁺ and Li⁺, which are well quantifiable by ICP–MS or ICP–OES. Prior to the use of these tracer ions, we checked whether they alter the enzymatic activities of trypsin, chymotrypsin, and pancreatic elastase in pure enzyme solutions or a small intestinal lavage as described above. We could confirm that the enzymes tested were not inhibited by the tracer ion concentrations used (data not shown). We then washed the intestine that had first been flushed with perfluorohexane with 2–5 ml of a spiked aqueous lavage buffer. Yet, the dilution of the lavage buffer with intestinal juice was not resolvable because the change in concentration of the tracer ions was smaller than the error of measurement. Volumes of less than 2 ml of spiked lavage buffer, on the other hand, could not be recovered from the intestine. As a consensus, we finally used a mixture of 500 μ l of Cs⁺- and Li⁺-traced aqueous buffer and a 10-fold excess of perfluorohexane to flush the intestine. The recovered aqueous portion of this wash (traced wash) contained a further fraction of intestinal fluid of which the dilution could be determined by measuring the tracer ion concentrations via ICP–MS or ICP–OES (see Eq. 2). The corresponding amount of undiluted intestinal fluid harvested in this step was 40 μ l (32–49 μ l) for fasted animals and 55 μ l (32–78 μ l) for unfasted animals.

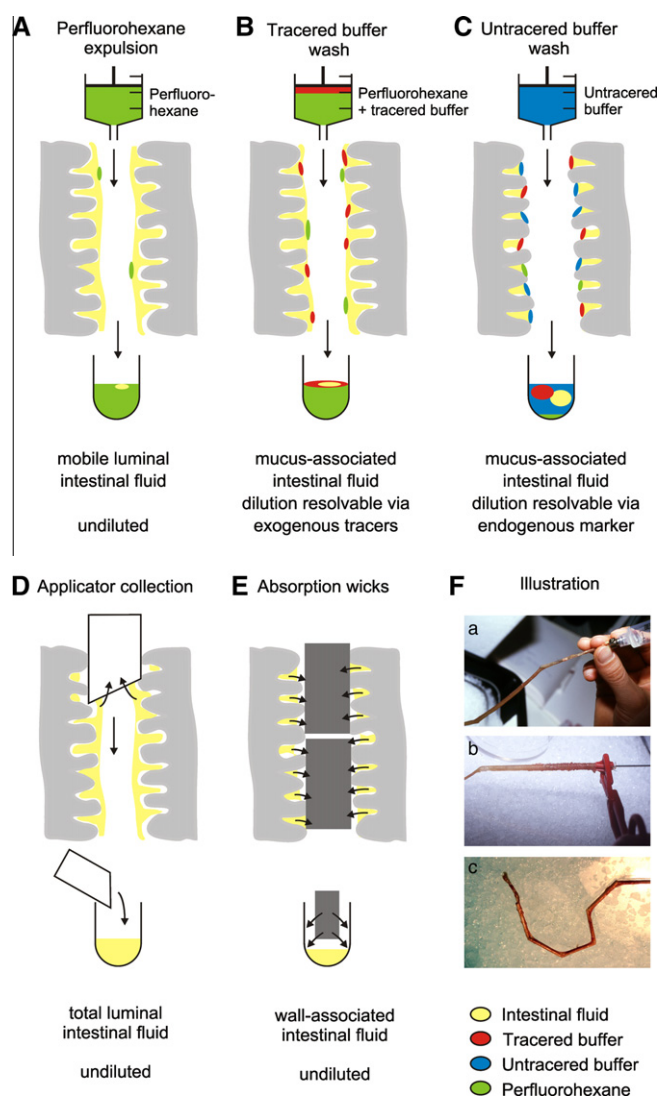


Fig. 1. Schematic representation of the three-tiered lavage and filter wick methods. (A–C) Three-tiered lavage procedure where perfluorohexane (green) is used to displace undiluted intestinal fluid (yellow) (A) before diluted intestinal fluid is collected by flushing with tracered physiological buffer (red) (B) or untracered physiological buffer (blue) (C). (D and E) Filter wick method where undiluted intestinal fluid is collected with a glass applicator (D) before wall-associated fluid is sampled with nylon filter wicks (E). (F) Illustration of perfluorohexane expulsion (a), glass applicator insertion (b), and positioned absorption wicks (c).

However, the instilled tracered buffer could be recovered only partially, and the total volume of undiluted intestinal fluid attainable with the glass applicator was still not achieved with the combined expelled fraction and tracered wash. To possibly elute more of the intestinal juice, we performed another wash of the small intestine with 5 ml of untracered physiological buffer. The collected eluate (untracered wash) comprised a mixture of perfluorohexane, intestinal fluid, and tracered and untracered buffer.

We conclude that by flushing the intestine with perfluorohexane, only a very mobile luminal fraction of intestinal fluid is eluted (Fig. 1). The aqueous washes of the three-tiered lavage extract some of the less mobile fluid probably associated with the mucus layer. The intestinal juice collected in the glass applicator presumably contains a major part of mucus-associated fluid in addition to the easily dislodged luminal contents. Further fluid that is retained more strongly by the mucus coat close to the epithelial cell wall is soaked up by the filter wicks that were placed directly into the intestine.

Histological analysis

To examine whether the overall architecture of the intestine had been damaged by the three-tiered lavage treatment, the medial region of the small intestine was analyzed histologically. Tissue samples taken from either fasted or unfasted animals that had been treated with the lavage technique were sectioned and dyed with PAS stain, and the overall architecture was compared microscopically with samples from unfasted animals whose intestines had not been treated with the three-tiered lavage (Fig. 2). There was no conspicuous difference, either between the treated and untreated small intestines or between animals that had been deprived of food before treatment and unfasted animals. We conclude that the lavage is a gentle method to elute the available luminal fraction of intestinal fluid and does not affect the mucosal lining cells. As demonstrated before [8], sampling of fluid with the glass applicator and filter wicks also provides a mild treatment without damaging the mucosa.

In vivo concentrations of digestive proteases, IgA, and mucin

To reveal the actual *in vivo* situation (i.e., the concentration of intestinal constituents that ingested substances must cope with), we first focused on samples where the intestinal fluid had not been diluted i.e., the perfluorohexane-displaced fluid, the fraction of intestinal fluid that had been isolated with the glass applicator, and the fraction extracted from the intestinal wall via filter wicks. To identify an analyte that might subsequently serve as a standard marker to calculate dilution factors of intestinal lavages, we determined the concentrations of enzymes that enter the intestinal fluid via the pancreatic duct from the luminal side (trypsin, chymotryp-

sin, and elastase) and of analytes that are secreted by the epithelium (IgA and mucin). Total protein was chosen as a general marker (Table 1).

The absolute concentrations of all analytes investigated were different in all fractions, and we observed either a steady increase of the analyte concentration from the luminal fractions toward the intestinal wall fraction (e.g., for total protein) or a hill-like distribution, with maximum values in the fraction collected with the glass applicator. This picture was similar for fasted and unfasted animals, and it raised the question which of the fractions of intestinal fluid was representative for the *in vivo* situation.

We first wanted to check whether the fractions differ only in their concentrations or also in their protein compositions. Therefore, we compared the relative analyte concentrations (i.e., the mg analyte/mg total protein) in all fractions (Fig. 3). The relative analyte concentrations in the perfluorohexane-displaced fluid were similar to those in the fraction collected with the glass applicator, but in nearly all cases they were higher than at the intestinal wall. These data allow the interpretation that the glass applicator fraction mirrors most closely the *in vivo* situation in the intestinal lumen in terms of the milieu that passing foreign substances encounter. The highly mobile luminal fraction, which is reflected in the perfluorohexane-displaced fluid, represents an analogously compounded, yet diluted (e.g., by drinking water), version of it. The composition of the wall-associated fluid, however, differs from that of the luminal fraction.

To investigate the extent to which intestinal lavages can reflect the *in vivo* situation, we checked whether the fluids isolated by the aqueous lavages with tracered or untracered buffer were comparable to the luminal fractions of undiluted intestinal fluid. Via the exogenous dilution markers, Li^+ and Cs^+ , the concentrations of the analytes in the tracered wash could be recalculated to the concentrations in undiluted fluid. The recalculated absolute concentrations (i.e., mg analyte/ml fluid) of all analytes lay in the range between the fractions isolated via perfluorohexane and the glass applicator (Table 1), and the relative concentrations (i.e., mg analyte/mg total protein) were also similar. For the untracered wash, recalculation of absolute concentrations was not possible at that point in time. However, here too the relative concentrations of most analytes were comparable to those of undiluted intestinal fluids (Fig. 4). Although there were some statistical differences between some fractions in fasted animals, the mean values and the variation of the data were overall in the same range. Thus, we conclude that the aqueous lavages eluted the intestinal solutes in a way that was representative for the undiluted situation. Chymotrypsin and IgA were the analytes that displayed the least differences in the relative concentrations of undiluted and lavage-based fluids (Fig. 4F and L); hence, these markers appear to be the most sensible endogenous dilution markers for murine intestinal lavages.

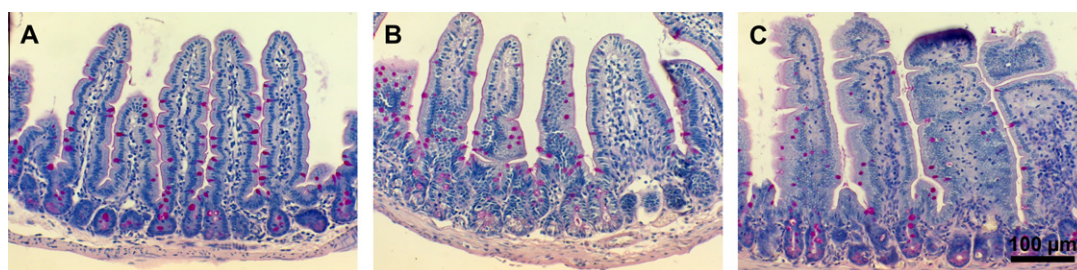


Fig. 2. Histological analysis of treated small intestines. Shown are photomicrographs of formalin-fixed and ParaPlast Plus-embedded 2- μm serial sections of the medial part of the small intestine stained with PAS dye. Intestines had either been treated with the three-tiered lavage (A and B) or not (C). Before treatment, animals were either deprived of food for 2.5 h (A) or not (B and C). For each treatment, a representative section of the small intestine is presented. Blue: cell membranes and nuclei; pink: mucus-rich compartments (glycocalyx and mucus layer, goblet cells).

Table 1
Concentrations of proteins in isolated intestinal fluid fractions.

Analyte	Nutritional condition	Three-tiered lavage				Collection and absorption	
		Perfluorohexane expulsion	Tracered wash		Untracered wash	Glass applicator	Absorption wicks
		Undiluted	Undiluted ^a	Diluted	Diluted	Undiluted	Undiluted
Total protein	Fasted	11.99 (8.38–15.60)	20.55 (14.90–26.20)	6.51 (3.37–9.65)	0.38 (0.25–0.50)	25.40 (23.35–27.44)	50.85 (43.50–58.20)
	Unfasted	12.99 (7.46–18.53)	21.19 (17.42–24.95)	10.58 (7.96–13.20)	0.72 (0.59–0.86)	31.16 (25.10–37.21)	53.48 (48.46–58.50)
Trypsin-like	Fasted	0.54 (0.23–0.86)	0.79 (0.52–1.05)	0.25 (0.12–0.39)	0.01 (0.01–0.02)	1.26 (1.11–1.41)	0.33 (0.23–0.42)
	Unfasted	0.61 (0.11–1.11)	1.21 (1.02–1.40)	0.62 (0.45–0.79)	0.04 (0.03–0.04)	1.20 (1.09–1.31)	0.51 (0.40–0.61)
Chymotrypsin-like	Fasted	1.20 (0.69–1.71)	1.07 (0.80–1.34)	0.32 (0.20–0.44)	0.02 (0.01–0.03)	2.18 (1.71–2.65)	0.61 (0.41–0.81)
	Unfasted	0.86 (–0.25–1.97)	1.36 (0.89–1.83)	0.70 (0.39–1.01)	0.04 (0.03–0.06)	1.60 (1.30–1.89)	0.81 (0.65–0.96)
Elastase-like	Fasted	0.34 (0.17–0.51)	0.26 (0.17–0.34)	0.08 (0.04–0.12)	0.01 (0.00–0.01)	0.54 (0.44–0.63)	0.12 (0.08–0.16)
	Unfasted	0.33 (–0.01–0.66)	0.41 (0.30–0.51)	0.21 (0.14–0.28)	0.02 (0.01–0.02)	0.57 (0.49–0.65)	0.18 (0.14–0.21)
Mucin	Fasted	4.10 (–1.67–9.86)	6.31 (–2.16–14.80)	2.65 (–1.90–7.20)	0.40 (–0.10–0.90)	4.45 (3.40–5.51)	3.34 (2.61–4.08)
	Unfasted	4.05 (–0.75–8.84)	5.68 (3.36–8.00)	2.96 (1.41–4.51)	0.63 (0.06–1.20)	6.24 (4.23–8.26)	2.75 (2.12–3.37)
IgA	Fasted	0.23 (0.07–0.39)	0.46 (0.35–0.57)	0.14 (0.09–0.20)	0.01 (0.01–0.01)	0.64 (0.46–0.82)	0.58 (0.48–0.68)
	Unfasted	0.12 (0.05–0.19)	0.34 (0.26–0.42)	0.17 (0.12–0.23)	0.01 (0.01–0.01)	0.32 (0.21–0.43)	0.52 (0.41–0.62)

Note. Data are given in mg/ml as means and ranges (95% confidence interval) of 6–10 animals.
^a Analyte concentration in undiluted intestinal fluid recalculated via exogenous dilution markers applying Eq. (2).

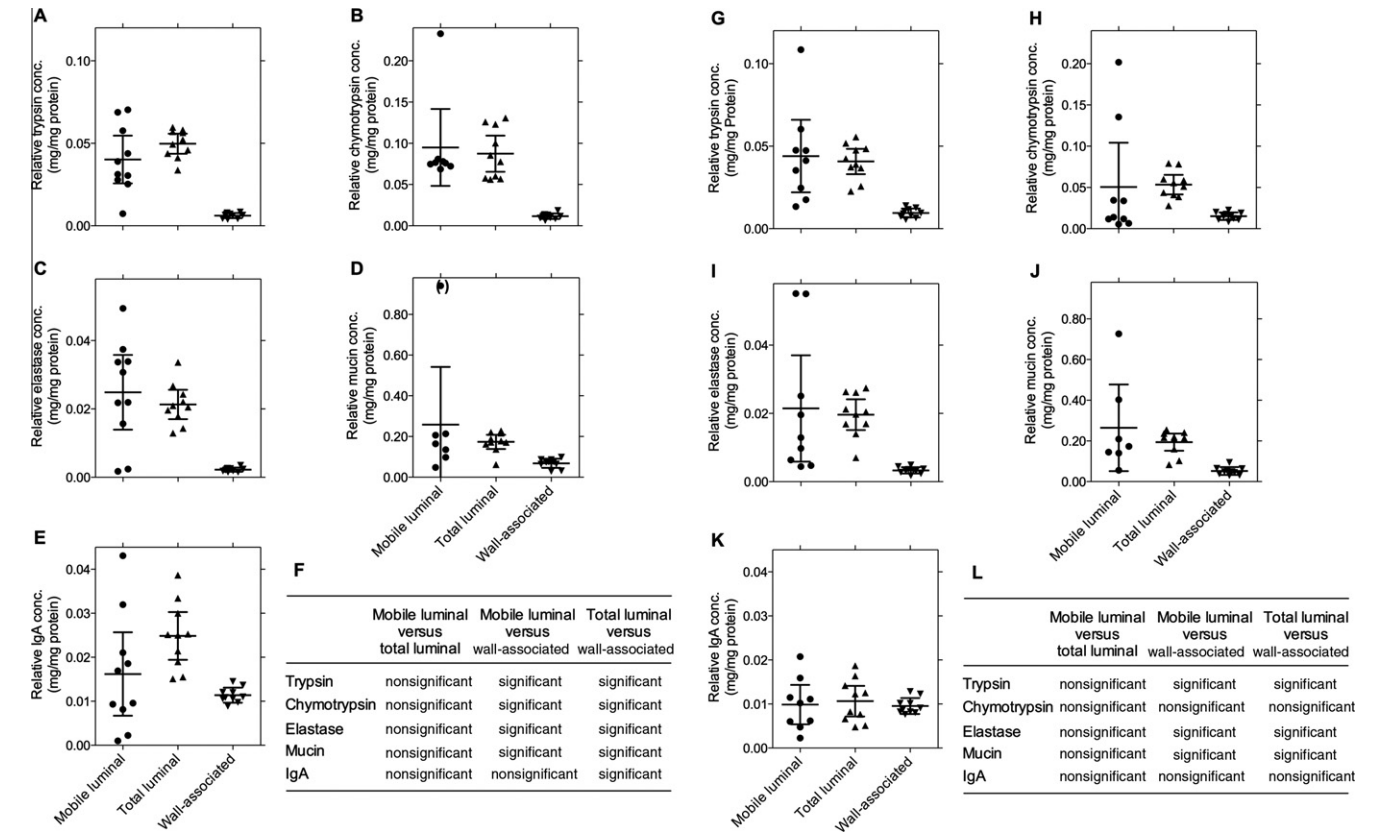


Fig. 3. Relative concentrations of intestinal solutes in differently harvested fractions of intestinal fluid. Relative concentrations (mg analyte/mg total protein) of trypsin (A and G), chymotrypsin (B and H), elastase (C and I), mucin (D and J), and IgA (E and K) in fractions of undiluted intestinal fluid isolated from fasted (A–F) and unfasted (G–L) animals are presented. The fraction of intestinal fluid isolated via the perfluorohexane expulsion represents the mobile luminal fluid, the fraction isolated with the glass applicator encompasses the total luminal content, and the fluid isolated by the filter wicks represents the intestinal wall-associated intestinal fluid. Statistical comparisons in panels F and L show whether the specific activities in the mentioned fractions are different from each other (one-way ANOVA, Bonferroni post hoc test, $P < 0.05$).

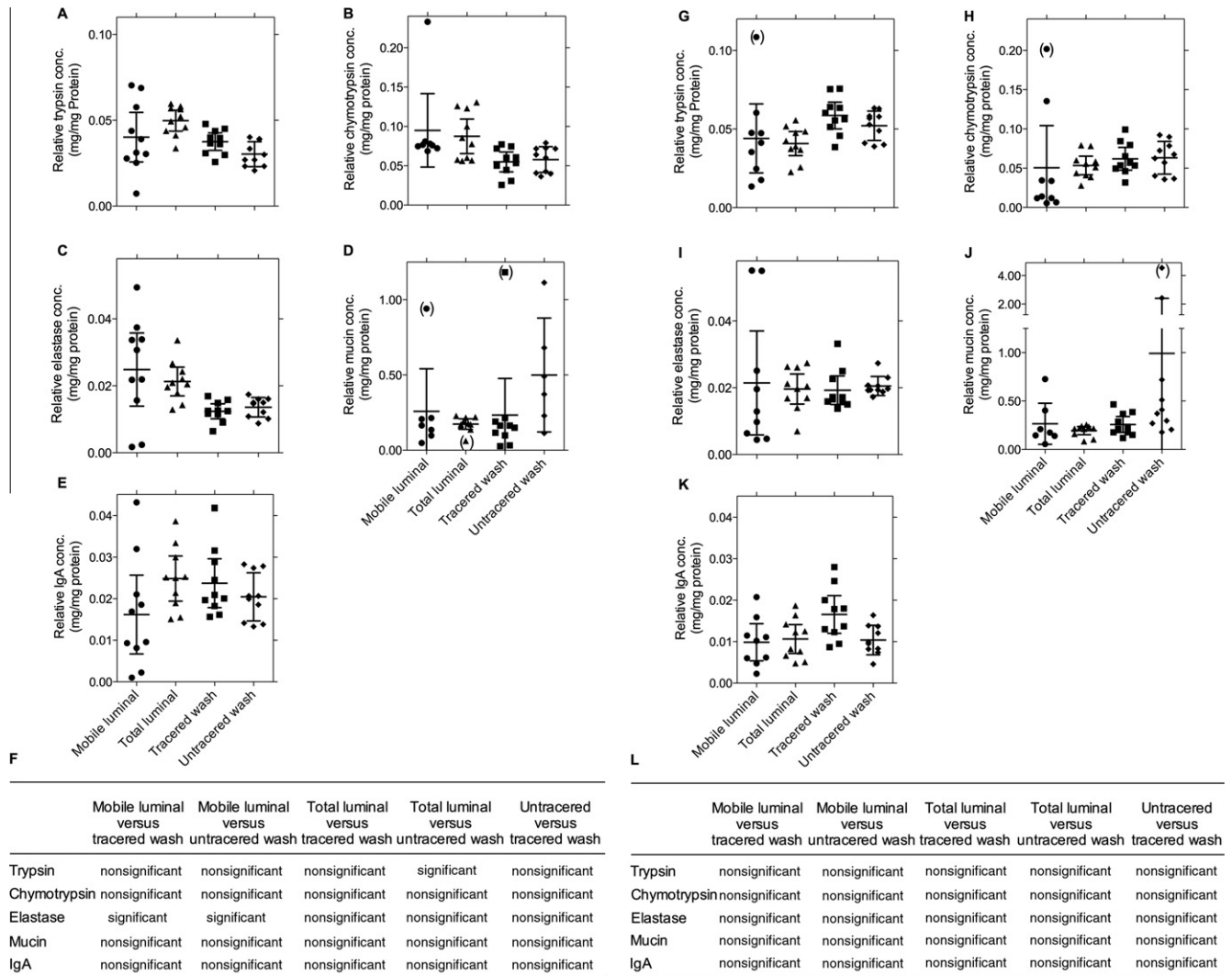


Fig. 4. Relative concentrations of intestinal solutes in undiluted intestinal fluid and lavage fluids. Relative concentrations (mg analyte/mg total protein) of trypsin (A and G), chymotrypsin (B and H), elastase (C and I), mucin (D and J), and IgA (E and K) in fractions of undiluted intestinal fluid isolated from fasted (A–F) and unfasted (G–L) animals are given. The fraction of intestinal fluid isolated via the perfluorohexane expulsion represents the mobile luminal fluid, and the fraction isolated with the glass applicator encompasses the total luminal content. Tracered buffer and buffer represent the respective lavage fluids of the three-tiered lavage. Statistical comparisons in panels F and L show whether the specific activities in the mentioned fractions are different from each other (one-way ANOVA, Bonferroni post hoc test, $P < 0.05$).

Finally we examined whether the total luminal fraction obtained with the glass applicator and the combined fractions of the three-tiered lavage yielded the same amount of each intestinal solute (Fig. 5). In the case of unfasted animals, both preparation techniques generated comparable yields of analyte. However, from the intestine of fasted animals, significantly lower amounts of the analytes—with the exception of mucin—were isolated by the lavage than with the glass applicator. Despite that fact, we would nevertheless recommend that fasted animals be used for isolation of intestinal juices simply because the variability in total and relative amounts was much lower (data not shown). In addition, for ex vivo/in vitro tests on the fate of ingested substances, the quantity of solutes is less important than the representativeness of the fluid. Besides, the enzymatic activity of undiluted intestinal juice is very high, and a small amount of undiluted fluid will be sufficient for numerous in vitro stability tests.

Discussion

The fate of substances such as foods, allergens, drugs, vaccines, pathogens, and anthropogenic xenobiotics in the digestive tract is

difficult to assess. Direct in vivo analysis requires sophisticated analytical technology [12] and may be restricted to special cases. Explanted live intestines [13] provide a promising alternative but have not been used for assaying the fate of ingested materials. In broad use are artificial in vitro systems in which a gastrointestinal transit is mimicked by a mechanical device that is filled with simulated gastric and intestinal fluids that are formulated according to standard recipes from a pharmacopeia [14]. Yet, such standardized fluids barely reflect the in vivo situation. It was shown that the three pancreatic enzymes typically used for simulated intestinal fluid—chymotrypsin, elastase, and trypsin—make up only one-third of the proteolytic activity in natural intestinal fluid and that diluted small intestinal fluid decomposes more rapidly by autolysis than undiluted preparations [3]. Other players, such as mucin and immunoglobulins, often are missing in such a setting. Thus, any faithful representation of the situation in the small intestines requires the use of natural intestinal fluid in its original composition and concentration.

By using different isolation methods, we could harvest such undiluted murine small intestinal fluid. However, each of the three preparations obtained in this way was different, either in terms of

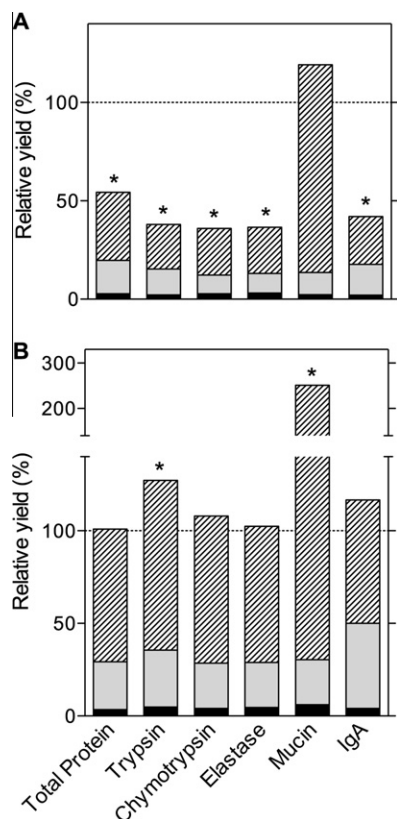


Fig. 5. Yields of intestinal solutes isolated via the three-tiered lavage from fasted and unfasted animals. Mean yields of each intestinal solute (total protein, trypsin, chymotrypsin, elastase, mucin, and IgA) obtained by the three-tiered lavage from fasted (A) and unfasted (B) animals were compared with the respective yields that had been obtained with the glass applicator (= 100%). The respective subquantities from the washes of the three-tiered lavage are indicated (black: perfluorohexane expulsion; gray: tracered wash; hatched: untracered wash). Cumulative yields (summed up over the entire three-tiered lavage process) that differ from 100% are indicated with an asterisk (*, one-sample *t* test, $P < 0.05$).

solute concentrations or with regard to composition and/or accessibility. Because we could rule out experimental artifacts such as inhibition of enzymatic activity and epithelial damage caused by the harvesting tools, we are confident that the three compartments indeed exist. This raises the question of which of those compartments reflects the actual *in vivo* situation that passing material must deal with. Because all preparations descend from the small intestinal lumen, and because there are no insurmountable physical barriers in the lumen it is clear that ingested substances may come into contact with either compartment, only the contact time may differ for different ingested materials. Thus, it is very difficult to designate a single preparation as being representative for the entire situation.

One preparation was obtained by flushing the small intestines with water- and lipid-immiscible liquid. This approach originated in the idea that a water- and lipid-immiscible liquid would form a bolus that could be pushed through the intestines like a highly flexible sphere. In practice, however, this did not work out. Viscous, highly coherent perfluorinated hydrocarbons formed that bolus but percolated so slowly that no luminal content was displaced. Only the least viscous perfluorohexane moved quickly enough to mobilize some liquid content, but all solid and viscous constituents remained in place. Therefore, we did not obtain the entire luminal content; rather, we obtained only the most mobile fraction of luminal constituents. A second preparation made use of a smoothed glass applicator with which we could skim off all of the loosely adherent material residing in the lumen. Finally, a third fraction

was accessible by placing filter wicks into the lumen. The suction of these wicks recovered this least mobile material that may have been trapped in the epithelial glycocalyx.

When comparing the absolute concentrations of the five analytes tested—trypsin-, chymotrypsin-, and elastase-like activities, mucin, and IgA—in the three undiluted preparations, it turned out that they were highest throughout in the glass applicator-harvested total luminal fraction. Thus, if an ingested substance withstands this preparation, it should also be resistant to the other two preparations. For that reason, the glass applicator-harvested total luminal fraction was set as reference.

To find out whether the perfluorohexane-expelled mobile fraction and the wick-absorbed wall fraction were simply diluted variants of the total luminal fraction or whether they differed in composition, the respective relative analyte compositions were compared. The statistical analyses showed that the wall fraction was different in terms of analyte composition. It obviously contains large amounts of non-analyte protein. Researchers who want to investigate luminal events that take place in close vicinity to the epithelial surface, therefore, should use filter wick-absorbed luminal content for their work. The perfluorohexane-expelled mobile fraction, on the other hand, had the same relative composition as, but only approximately half the analyte concentration of, the total luminal fraction. It represents a diluted solution of the total luminal preparation. The fact that the epithelium-borne analytes are not significantly enriched in the mobile fraction confirms our histological observation that the perfluorohexane treatment does not harm the epithelium. Only a slight irritation of the goblet cells (if any at all) may have occurred by the high density and the surface-active properties of the hydrocarbon [15], as indicated by a slightly reduced number of mucus-filled goblet cells detected in the histological sections and by the somewhat higher coefficient of variation in the mucin data of perfluorohexane-treated animals compared with untreated animals. Because perfluorohexane expulsion yields only approximately one-tenth of the amount one can harvest with the glass applicator, this technique might not be of general interest. However, we could demonstrate with this method that the most mobile fraction is already diluted *in vivo* (e.g., by drinking water), a fact that should be kept in mind when human small intestinal juice is sampled by intubation and aspiration. Such mobile fractions may contain approximately two times less analyte than what is actually present in the lumen of the small intestine.

Although the glass applicator-based procedure allows harvesting undiluted intestinal fluid in large yields, the technique is laborious and requires custom-made equipment. Yet, many assays do not necessarily require undiluted intestinal fluid if only the exact dilution factor of an intestinal fluid sample is known. Therefore, we wanted to know whether exogenous and/or endogenous dilution markers can be used to recalculate the original fluid concentration.

Li^+ has been used before as exogenous dilution marker in mucosal washes [16,17]. It was shown to be barely taken up in the course of an intestinal lavage [18], and the recovery of the ions from stool and mucus substances was reported to be excellent [19]. Therefore, we tested Li^+ along with Cs^+ as nonphysiological tracers for the determination of dilution factors in intestinal lavages. In our hands, however, the alkali ion tracers did not live up to the expectations. To allow a reliable recalculation of the dilution, the amount of tracered wash fluid is restricted to approximately 10 times the amount of undiluted intestinal juice, which translates into wash buffer volumes of less than 2 ml. With this little buffer, we recovered only minimal amounts of the administered ions and most fluid remained trapped in the gut. Therefore, we combined the perfluorohexane expulsion with a tracered lavage (step 2 in the three-tiered lavage) and were able to harvest a reasonable portion of tracered buffer-diluted intestinal fluid. After recalculating

to the undiluted status, the analyte concentrations were similar to those obtained by the glass applicator preparation that supported the choice of the glass applicator preparation as reference.

Although workable for intestinal fluid harvesting and determining in vivo analyte concentrations, the use of tracer-spiked binary lavage buffer is not attractive for routine use. It is tricky to apply and requires heavy equipment for the quantitation of the tracer ions. Therefore, we wanted to know whether aqueous lavages liberate the luminal constituents uniformly such that one of our analytes can be used as endogenous dilution marker in tracer-free preparations. We found that this was indeed the case. The relative chymotrypsin and IgA content in any wash or expelled fraction was not significantly different from that in the glass applicator preparation, rendering these analytes valid endogenous dilution markers. Moreover, the yields after aqueous intestinal lavage were comparable to those obtained with our glass applicator reference procedure.

Because diluted intestinal fluid was shown to decompose faster than undiluted fluid [3], we recommend glass applicator-harvested preparations for any type of long-term analysis. For short-term proteolysis assays [20] and other fast experiments that require knowledge of the sample dilution factor but not of the undiluted intestinal fluid itself, the use of an aqueous lavage followed by the determination of the IgA and chymotrypsin content and subsequent recalculation of the dilution factors on the basis of the data we provide in Table 1 should be sufficient.

Besides these technological aspects, our study revealed some interesting physiological findings. The wall fraction, although rich in protein and similar to the luminal fraction in terms of the mucin and IgA content, contains surprisingly little pancreatic proteases. This lack of proteolytic activity could be due to an inability of the enzymes to enter the glycocalyx, similar to what was found for nanoparticles [21], or the pancreatic enzymes adhere so tightly to the glycocalyx that they could not be released by the suction of the wick. The latter interpretation fits well with the concept of membrane digestion proposed by Ugolev [22] that divides the digestive process into a “cavital digestion” in the lumen and a “membrane digestion” performed by adsorbed enzymes. It is also supported by findings of Goldberg and colleagues [23], who reported a noncovalent binding of trypsin and chymotrypsin to intestinal lining cells. If this holds true, our current understanding of the intestines as a tubing homogeneously filled with digestive enzymes may need to be reappraised.

Acknowledgments

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