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Parallel Determination of Gut Permeability in Man with M_r 400, M_r 1500, M_r 4000 and M_r 10 000 Polyethylene Glycol[1])

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Summary: Polyethylene glycol has been in use for a number of years for the assessment of gut permeability. The methods so far employed are usually limited to polyethylene glycols in the low relative molecular mass range (up to M_r 1300). We developed a method for the simultaneous determination of gut permeability to M_r 400, M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol, by applying a single oral dose of an appropriate mixture of these polyethylene glycols. After extraction from 24 h-urine, M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol were quantified by size exclusion chromatography, while M_r 400 polyethylene glycol was determined by reversed phase chromatography. The detection limit of polyethylene glycol in the relative molecular mass range between M_r 1500 and M_r 10 000 was found to be 0.2 mg/l urine, and the detection limit of M_r 400 polyethylene glycol 5 mg/l urine. Recovery of the polyethylene glycols (N = 6) were 86.6% (CV: 4.8%) for M_r 400, 94.1% (CV: 7.2%) for M_r 1500, 97.1% (CV: 5.5%) for M_r 4000 and 97.4% (CV: 5.6%) for M_r 10 000.

No significant difference was found between the excretion rates in 24 h-urine of M_r 400 and M_r 1500 polyethylene glycols in patients with *Crohn*'s disease (M_r 400: 34.4 ± 5.5%; M_r 1500: 5.22 ± 2.27%; mean ± SEM, N = 10) and healthy controls (M_r 400: 33.6 ± 3.2%, M_r 1500: 1.09 ± 0.26%; N = 21). The excretion rate of M_r 4000 polyethylene glycol was markedly higher in patients with *Crohn*'s disease (0.462 ± 0.177%) than in healthy controls (0.049 ± 0.012%, $p < 0.05$). M_r 10 000 polyethylene glycol was detected in the urine of only one out of the 21 healthy controls, but in nine out of the ten patients with *Crohn*'s disease ($p < 0.001$). These results suggest a change not in number but rather in selectivity in permeability of high M_r molecules.

Introduction

Changes in the permeability of the gut mucosa have been the object of increasing interest during recent years, especially in patients with chronic inflammatory bowel diseases. Until now, almost all the compounds used to measure the intestinal permeability have been of low relative molecular mass (M_r 180–M_r 380). These have been mannitol (1) and rhamnose (2), frequently in combination with a disaccharide such as cellobiose (3, 4) or lactulose (5–7). In many cases, radioactive substances such as ^{51}Cr-EDTA (8–10), ^{99}Tc-DTPA (11), ^{14}C- or ^{3}H-labelled organic compounds (8, 12) have been used. Commonly used marker substances of high relative molecular mass are polyethylene glycols in the range between M_r 400 and M_r 600 (13–19) or around M_r 1300 (20–22).

Thus, intestinal permeability has been measured with either only low-M_r substances, or one containing radionuclides. A non-invasive test permitting the simultaneous determination of human gut permeability to compounds in both the low and high relative molecular mass range not containing radioactive components would be desirable.

The ideal substance must be neither toxic nor metabolised by either man or the intestinal flora. Polyethylene

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glycol meets all these demands (18), but its poor separability by reversed phase high performance liquid chromatography (RP-HPLC), and its unsatisfactory volatibility in gas chromatography have so far limited its use to the maximum relative molecular mass of M_r 1300 (18, 20, 23, 24–26). With the exception of polyethylene glycol M_r 3000 and M_r 4000, which were determined by open gel chromatography (27–29) or by a turbidimetric procedure (30), no high M_r polyethylene glycols have been used as markers in the systematic assessment of permeability in man to our knowledge.

In this study, measurement of the simultaneous absorption of the four M_r 400, M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycols has been made possible by optimizing the percentage of each relative molecular mass range in one orally administered mixture. The method was tested for reproducibility and applicability. The permeability measured in a group of patients with *Crohn*'s disease (N = 10) was compared with that of a group of healthy controls (N = 21).

Materials and Methods

Reagents and instrumentation

Chloroform p. a., methanol p. a. and M_r 400, M_r 4000, M_r 10 000 polyethylene glycols were purchased from Merck, Darmstadt, Germany. M_r 1500 polyethylene glycol was obtained from Serva, Heidelberg, Germany. Teflon filters (0.45 µm) were supplied by Aldrich, Steinheim, Germany.

All solvents were removed in a vacuum centrifuge (Bachhofer, Reutlingen, Germany). The separation was carried out on a semipreparative column (500 mm × 8 mm), filled with Lichrospher Diol 5 µm 100 Å (Merck, Darmstadt). This column was protected by a pre-column (20 mm × 4 mm), filled with the same material. M_r 400 polyethylene glycol was separated on a column (250 mm × 4 mm) containing Nucleosil 7C8 (Macherey-Nagel, Düren, Germany). All polyethylene glycol fractions were quantified by measuring the differential refraction index (type ERMA 7512, ERC, Alteglofsheim, Germany). The signals of this detector were evaluated by an integrator (type 2000 D, Merck, Darmstadt, Germany).

Polyethylene glycol application and extraction

Each test subject ingested 2 g M_r 400, 1.5 g M_r 1500, 5 g M_r 4000 and 10 g M_r 10 000 polyethylene glycol, all dissolved together in 100 ml of water; this solution was given as a drink after breakfast. Urine was collected during the next 24 hours. Hundred ml of the homogenised urine were stored at −20 °C and analysed within 6 months. For analysis, two 45 ml volumes of the urine were centrifuged for 10 minutes at 1000 *g*. Clear supernatant (40.0 ml) was extracted with 3.0 ml chloroform by vigorous shaking (2 min). The lowest, emulsified, chloroform/urine layer was transferred into centrifugation tubes. The urine was extracted once more with 2.0 ml chloroform. After centrifugation, the two chloroform/urine emulsions were combined. The supernatant urine was used for determination of M_r 400 polyethylene glycol.

Size exclusion chromatography of M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol

The chloroform/urine emulsion was centrifuged (2500 *g*, 20 min at 4 °C) and the clear chloroform layer washed twice with distilled water. After removal of water, the remaining chloroform layer was weighed. The loss of polyethylene glycol during the extraction procedure was determined by calculating the final volume of chloroform against the start volume. The M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycols remaining in the residue after evaporation of chloroform were extracted with 400 µl of HPLC solvent (methanol/water, 66 + 34, by vol.) for ten minutes in an ultrasonic bath (50 °C). The resulting suspension was filtered through a teflon membrane filter (0.45 µm) and 200 µl of this solution were applied to the HPLC at a flow rate of 1 ml/min (method A).

Reversed phase chromatography of M_r 400 polyethylene glycol

Four ml of the urine, remaining after extraction with chloroform, were evaporated. For complete dissolution of M_r 400 polyethylene glycol, the residue was extracted with 1 ml of methanol in an ultrasonic bath at 50 °C for 30 min and centrifuged (2500 *g* for 5 min). Fivehundred µl of the clear supernatant were evaporated to dryness and the residue redissolved in 400 µl mobile phase (methanol/water 20 + 80, by vol.). Hundred µl of this solution were quantified by reversed phase chromatography (Nucleosil 7C8, 1 ml/min, method B, modification of a method described in l. c. (24)). To minimize difficulties during the determination of M_r 400 polyethylene glycol (fig. 6), peak area of the M_r 418 compound (26.09 min) was used for calculation of M_r 400 permeability. This compound has shown the lowest interference with the initial background peak and the lowest difference to the theoretical relative molecular mass ratio M_r 400. Recovery was calculated as the quotient of the peak area of the urine-extracted compound and the peak area of the fraction in standard solution, directly employed in HPLC.

Patients and healthy volunteers

To test the applicability of this procedure, the permeability of the gut to all four polyethylene glycol molecular weight fractions was determined in a group of healthy controls (N = 21) and a group of patients with *Crohn*'s disease (N = 10). The diagnosis of *Crohn*'s disease was confirmed by X-ray and/or endoscopy findings. The average *Crohn*'s disease activity index (CDAI as reported by *Beck* (31)) of the patients with *Crohn*'s disease was 140 ± 21. None of the patients had undergone resection; their average age was 39.7 ± 4.4 years and their average body weight 70.2 ± 7.7 kg (mean ± SEM).

The members of the control group did not differ from those of the patient group in either age (37.0 ± 2.9 a) or weight (72.0 ± 4.34 kg), nor was there any difference in sex distribution. Creatinine clearance was normal in all subjects.

Mass ratio of ingested M_r 400, M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycols

The mass ratios of the different polyethylene glycols in the mixture employed were optimized in such a way as to obtain comparable concentrations of M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol in the urine samples. In this way it was possible to quantify these three fractions in a single chromatographic analysis. The amount of M_r 10 000 polyethylene glycol employed was limited to 10.0 g because of the laxative effect of this compound. The ingested amount of M_r 400 polyethylene glycol was four times as large as M_r 1500 polyethylene glycol because a less sensitive method of analysis was used.

Calibration, linearity, reproducibility, recovery, stability and detection limits

Before polyethylene glycol ingestion, urines of both groups were checked for the appearance of disturbing peaks in the chromato-

grams at coincident retention times. The pooled polyethylene glycol stock solution in urine had the following concentration: M_r 400: 2.0 g/l; M_r 1500: 500 mg/l; M_r 4000: 200 mg/l and M_r 10 000: 20 mg/l. This solution was diluted with urine 1 : 2, 1 : 4, 1 : 10, 1 : 50, 1 : 100 and 1 : 200. These diluted standard solutions were apportioned, stored at −20 °C, and used for the determination of calibration curves, reproducibility, stability and detection limits. Recovery after extraction from urine was determined by calculating the peak area of each polyethylene glycol fraction against the peak area of standard solutions dissolved in the appropriate mobile phase. Reproducibility and stability were checked in aliquots over a period of six months.

Statistics

All results are indicated as mean values ± standard error of mean ($\bar{x}$ ± SEM). *Wilcoxon*'s rank sum test was used to assess significances between the two groups. Results obtained measuring the permeability of M_r 10 000 polyethylene glycol were not suitable for *Wilcoxon*'s rank sum test, because no M_r 10 000 polyethylene was detected in the control group in 9 out of 10 urines. These results were compared by the χ^2-test.

Results

Chromatographic separation and extraction of different polyethylene glycol fractions

Organic biopolymers, usual matrices for separation of constituents according to their relative molecular mass, showed after a few chromatographic runs with samples gained by the described procedure a fast increase in pressure over the maximum limit. Because of its porosity (about 100 Å) and its pressure stability, semipolar reversed phase material (Diol) proved highly suitable for size exclusion chromatography, when all attraction phenomena of polyethylene glycol to the stationary phase were suppressed. In an attempt to find the best mobile phase, the methanol/water solvent was optimized. Figure 1 shows the separation of the four polyethylene glycol-fractions of the methanol/water ratio. The capacity factor k' was used as a measure of separation. The capacity factor k' is defined as

$$k' = \frac{t_r - t_m}{t_m},$$

where t_r is the retention time of the analysed molecule fraction and t_m the retention time of a non-retarded solvent molecule since injection.

The capacity factors of the four polyethylene glycol M_r fractions employed were measured with four different compositions of the mobile phase. A regression analysis of these results (polynome, second grade) was made to identify the optimal point of separation for all four polyethylene glycol fractions, which was at the maximum of

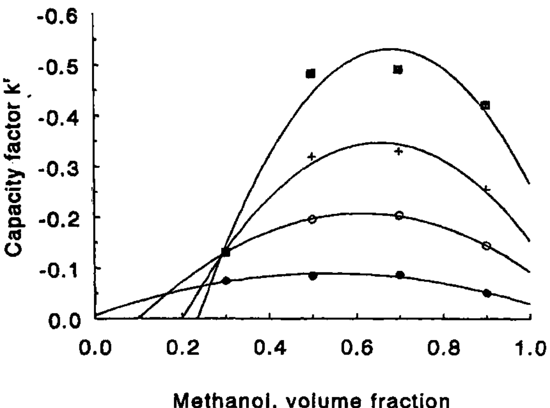
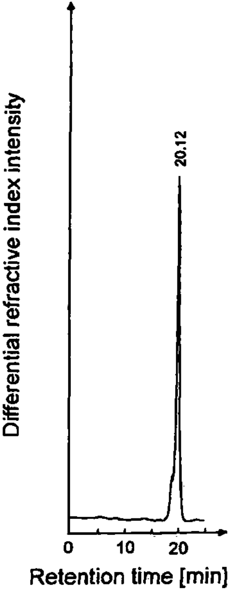


Fig. 1 Capacity factor k' of four polyethylene glycol fractions (●: M_r 400, ○: 1500, +: M_r 4000 and ■: M_r 10 000) on a Lichrospher Diol 5 µ column (500 mm × 8 mm, flow: 1 ml/min) as a function of the volume fraction of methanol in mixture with water.

the approximated function (fig. 1). Optimal conditions were found to differ for each M_r fraction, ranging from 54 + 46 (methanol/water) for M_r 400 polyethylene glycol to 68 + 32 for M_r 10 000 polyethylene glycol. Satisfactory simultaneous separation of all four fractions was obtained at a mixture of 66 + 34. Under these conditions, there was no increase in pressure over more than 100 runs with samples gained by the described procedure.

Under the given conditions of chloroform extraction, migration of M_r 400 polyethylene glycol from the urine into the organic layer was below the detection limit. This lack of extractability of M_r 400 polyethylene glycol made a separate analysis necessary. M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol, on the other hand, were enriched completely in the chloroform layer by twofold extraction of the organic solvent. A third chloroform extraction contained no detectable traces of any of this three polyethylene glycol fractions.

No disturbing signals at relevant retention times for M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol were obtained after extraction (method A) of urines containing no polyethylene glycol (fig. 2). Figure 3 shows the chromatogram of an evaporated chloroform extract of a urine stocked with 500 mg/l of each of the four polyethylene glycol fractions. A chromatogram of a standard solution of M_r 400 polyethylene glycol (2 g/l) is shown in figure 4. Urine without polyethylene glycol showed no disturbing peaks at the retention time of M_r 418 (fig. 5). After oral administration of the described polyethylene glycol mixture, the content of M_r 400 polyethylene glycol in the excreted urine could be quantified (fig. 6). Permeability of M_r 400 polyethylene glycol was calculated as the permeability of M_r 418 polyethylene glycol (26.09 min in fig. 6).



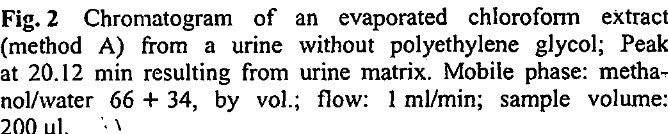


Fig. 2 Chromatogram of an evaporated chloroform extract (method A) from a urine without polyethylene glycol; Peak at 20.12 min resulting from urine matrix. Mobile phase: methanol/water 66 + 34, by vol.; flow: 1 ml/min; sample volume: 200 µl.

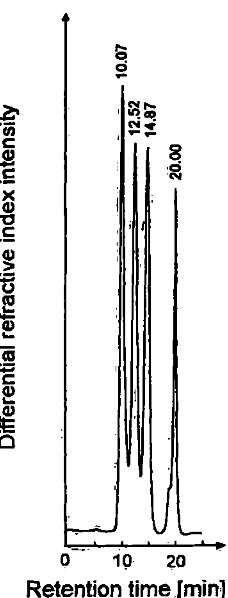


Fig. 3 Chromatogram of an evaporated chloroform extract (method A) from urine; the urine was spiked with 500 mg/l each of M_r 400, M_r 1500 (14.9 min), M_r 4000 (12.5 min) and M_r 10 000 (10.1 min) polyethylene glycol. Peak at 20.00 min resulting from urine matrix. Mobile phase: methanol/water 66 + 34, by vol.; flow: 1 ml/min; sample volume: 200 µl.

The detection limit of method A, defined by a signal-to-noise-ratio of 5 : 1, was determined for M_r 1500, M_r 4000 and M_r 10 000 polyethylene glycol to be 0.2 mg/l each. The detection limit for method B (M_r 400 polyethylene glycol) was 5 mg/l, which reflected the lower enrichment of polyethylene glycol by this method. The recovery of polyethylene glycols (N = 6) in frozen urine measured on different days was 86.6% (CV: 4.8%) for M_r 400, 94.1% (CV: 7.2%) for M_r 1500, 97.1% (CV: 5.5%) for M_r 4000 and 97.4% (CV: 5.6%) for M_r 10 000. The amount of polyethylene glycol proved to remain stable over a storage time of up to six months.

Permeability in patients with *Crohn*'s disease and in healthy controls

The excretion of M_r 400 polyethylene glycol by patients with *Crohn*'s disease (34.4 ± 5.5%, mean ± SEM) did not differ from that in the control group (33.6 ± 3.2%). There was also no difference of the relative amount of M_r 1500 polyethylene glycol in the permeated urine in patients with *Crohn*'s disease (5.22 ± 2.27%) as compared with that of the healthy controls (1.09 ± 0.26%, $0.05 < p < 0.10$). The excretion of M_r 4000 polyethylene glycol was significantly higher in patients with *Crohn*'s disease (0.462 ± 0.177%) than in the control group (0.049 ± 0.012%, $p < 0.05$). M_r 10 000 polyethylene glycol was detectable in the urine in only one of the 21 healthy controls (detection limit at 0.2 mg/l), but in nine of the patients with *Crohn*'s disease (0.229 ± 0.070‰, $p < 0.001$).

With the employed amounts of polyethylene glycols, laxative effects were neither observed in the group of patients with *Crohn*'s disease nor in that of healthy volunteers. This fact corresponds well with results published by other authors (18, 19, 21, 23).

Discussion

Polyethylene glycol was first used as a marker for measuring the permeability of the gut by *Chadwick* (18). Several other authors recognized the importance of the relative molecular mass of this permeability marker, but due to analytical constraints employed a maximal relative molecular mass of only M_r 1300 (20, 23–26). Polyethylene glycol, having a relative molecular mass of up to M_r 4000, was employed in only two studies, and analysed with the time-consuming open gel chromatography (27, 28). The new method described readily allows the simultaneous determination of gut permeability to polyethylene glycols ranging from M_r 400 to M_r 10 000 ingested simultaneously. Due to the urine sampling time (24 h), this procedure may serve to assess the overall permeability of compounds over a wide relative molecular mass range from the whole intestine into the systemic blood stream.

Poor pressure stability of the gel columns and the possibility of microbiological degradation make it difficult to work with those organic polymers often used as a separation matrix for size exclusion chromatography. Silica,

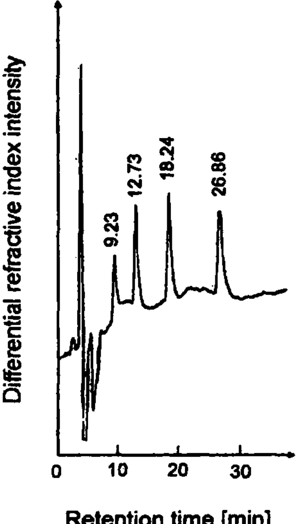


Fig. 4 Chromatogram of a standard solution of M_r 400 polyethylene glycol (0.1 g/l). Mobile phase: methanol/water 20 + 80, by vol.; flow: 1 ml/min; sample volume: 100 µl. Single peaks are: 9.23 min: M_r 286; 12.73 min: M_r 330; 18.24 min: M_r 374; 26.86 min: M_r 418 polyethylene glycol.

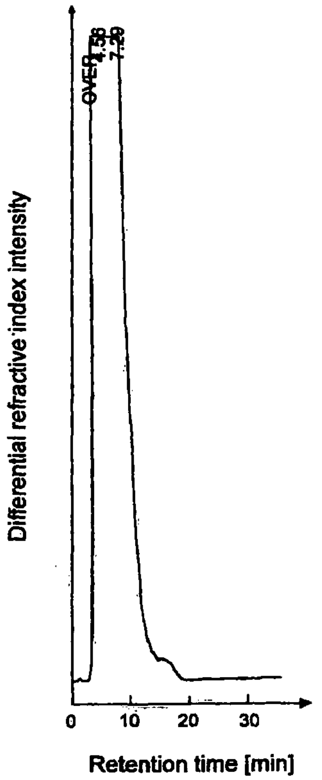


Fig. 5 Chromatogram of a methanolic extract (method B) of urine from a patient with *Crohn*'s disease without polyethylene glycol (method A). Mobile phase: methanol/water 20 + 80, by vol.; flow: 1 ml/min; sample volume: 100 µl.

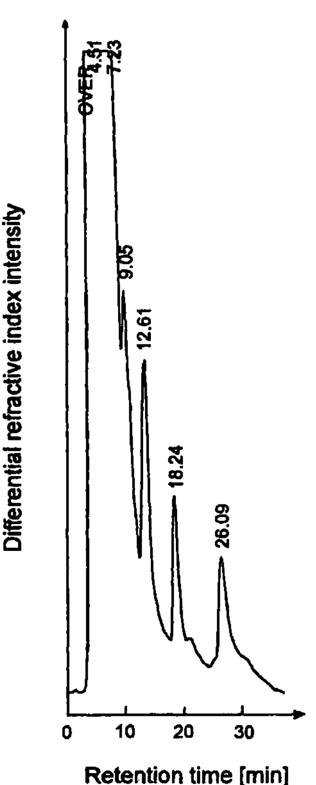


Fig. 6 Chromatogram of a methanolic extract (method B) of urine from the same patient with *Crohn*'s disease mentioned in figure 5 after ingestion of polyethylene glycol mixture. Mobile phase: methanol/water 20 + 80, by vol.; flow: 1 ml/min; sample volume: 100 µl. Single peaks are: 4.51 min and 7.23 min: initial background peaks; 9.05 min: M_r 286; 12.61 min: M_r 330; 18.24 min: M_r 374; 26.09 min: M_r 418 polyethylene glycol.

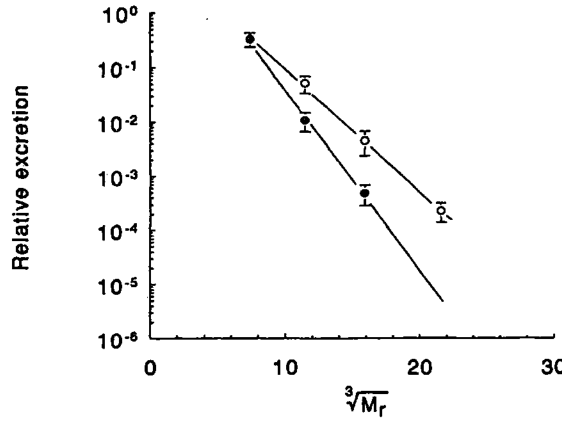


Fig. 7 Correlation between relative excretion of ingested M_r 400, M_r 1500, M_r 4000 and M_r 10000 polyethylene glycol and the molecular diameter, expressed as the cube root of the relative molecular mass M_r, in healthy controls (●, N = 21) and in patients with *Crohn*'s disease (○, N = 10). Vertical bars indicate SEM.

which does not have these disadvantages, is porous not only in its natural, but also in a modified state. This porosity makes it suitable for size exclusion chromatography. To obtain a range for optimization of the solvent, a semipolar Diol-phase was used as stationary phase. In order to be able to utilize the size exclusion effects, the strength of the mobile phase was adjusted so as to suppress all surface (adsorption and reverse phase) effects.

Mixtures of acetone, acetonitrile, tetrahydrofuran and methanol with water were all suitable for separation of

the polyethylene glycol fractions, but the mobile phase used was optimized with methanol as the organic component (fig. 1), resulting in high detection limits, reflecting the low refraction index of methanol.

Because of confounding elements in the urine, a method for the determination of M_r 400 polyethylene glycol by reversed phase-HPLC developed earlier (24), had to be modified.

Some authors have used M_r 400 polyethylene glycol for determination of gut permeability under comparable conditions (16, 19, 24, 32). Their results correspond well, in terms of order of magnitude, with the values determined in this study, although the amount of M_r 400 polyethylene glycol ingested was considerably higher (up to 5 times) than in the present study. Thus, the uptake and excretion of M_r 400 polyethylene glycol does not seem to correlate with the amount ingested.

The use of polyethylene glycol fractions with a molecular weight of between M_r 400 and M_r 1300 as permeability markers resulted in the detection of 20–50% of the ingested polyethylene glycol in the urine, even in persons with no signs of any bowel disease (14–16, 19, 21, 33). Among others, the high excretion rate of ingested polyethylene glycol may be the reason for the contradictory results obtained when comparing permeability in patients with *Crohn*'s disease and in healthy controls – the permeability to polyethylene glycol of less than M_r 1300 in the patients was found to be comparable (16, 19), higher (14, 33) or lower (15, 21) than in controls.

The importance of employing polyethylene glycols with $M_r > 1500$ is underscored by comparing the renal excretions of M_r 4000 and M_r 10000 polyethylene glycol in patients with *Crohn*'s disease and in healthy controls. The excretion of M_r 4000 polyethylene glycol in the patients was found to be ten times as high as in the controls. M_r 10000 polyethylene glycol was detected in the urine of only one out of 21 persons in the control group, but in that of nine out of ten in the *Crohn*'s disease group.

When using polyethylene glycol to investigate changes in intestinal permeability relating to certain bowel diseases, it may be important to be aware of the significance of the M_r of polyethylene glycol used. A decisive factor for the permeation of any compound through the epithelium of the mucosal barrier in the gut is the spatial extent of the molecule. The average diameter of this molecule is directly related to the cube root of relative molecular mass. In any case, the correlation between the diameter and the cube root of the relative molecular mass does not depend on the exact shape of the molecule. Doubling the average diameter of a molecule requires an eight-fold increase in molecular mass.

A search for the mathematical function best able to express the correlation between the diameter of the molecule and excretion showed the best empiric approximation to be the exponentional one:

$$\text{relative excretion} \sim b^{(\text{molecular diameter})}$$

and, thus,

$$\text{relative excretion} \sim b^{(\sqrt[3]{M_r})},$$

where b is the basis of the exponential function and M_r the relative molecular mass of the permeating molecule. This proportionality can also be expressed as an equation by introducing the proportionality factor k:

$$\text{relative excretion} = k \cdot b^{(\sqrt[3]{M_r})}.$$

Figure 7 shows the very good linearity of this correlation in both the healthy controls ($r = 0.9973$) and the patients with *Crohn*'s disease ($r = 0.9990$). The relative permeability clearly decreases more rapidly with increasing molecular diameter (expressed as the cube root of relative molecular mass) in the controls as compared with that of patients with *Crohn*'s disease.

The respective values calculated from permeability means of both groups for b and k were 0.595 and 1750 in patients with *Crohn*'s disease, and 0.464 and 8620 in healthy controls. In the above equation, the change in gut permeability is expressed mainly by the variable b. A rapid change in permeability with increasing M_r results in only a slight change of b. This slow change of b is caused by the high exponent, which is a function of the relative molecular mass. Therefore a slight change in b signifies a clear change in permeability. Means for b are 0.579 ± 0.019 for the patient group and 0.423 ± 0.019 for the group of healthy controls ($p < 0.001$).

Further investigations will be needed to show whether the parameters of the above regressed equation (especially b) can serve as factors for the assessment of inflammatory bowel diseases.

Figure 7 shows that, as compared with healthy controls, the percentage permeability in patients with *Crohn*'s disease was changed only slightly in the low, but markedly in the high molecular range. This may suggest an altered selectivity of leaky places in the mucosal gut barrier rather than a change in their number.

The determination of permeability of high M_r compounds in other intestinal diseases might also be impor-

tant because of their potentially antigenic effects. When investigating the relative molecular mass distribution of endotoxins in human feces, for example, we were able to identify endotoxins ranging between M_r 1500 and M_r 25000 (34). The fact that this range overlaps with that of the polyethylene glycol used in the present study (M_r 400–M_r 10000) underscores the importance of measuring permeability in the high-M_r range.

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