Bacteria, bile salts, and intestinal monosaccharide malabsorption

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SUMMARY Intestinal monosaccharide transport was studied in a series of rats with a self-filling jejunal blind loop using 3mM arbutin (p-hydroxyphenyl-B-glucoside) or 1mM D-fructose as substrate in vitro and 10 mM arbutin or 5mM D-fructose in vivo. These results were compared with changes in the bacterial flora and state of conjugation of intraluminal bile salts in those animals. Observations were also made of the microscopic and ultrastructural appearances of the small-intestinal epithelium.

In the small intestine of blind-loop rats intestinal monosaccharide transport is impaired, and in vitro is most marked in the blind loop, less so in the efferent jejunum, and not significantly altered in the afferent jejunum. A similar pattern of disturbed monosaccharide absorption was demonstrated by perfusions in vivo. The degree of the transport defect correlates closely with the luxuriance of the anaerobic flora, which averaged 10⁵ per millilitre in the blind loop, 10⁷ in the efferent jejunum, and 10⁸ in the afferent jejunum. A similar pattern of abnormality of bile salt conjugation occurred. In the blind loop the ratio of free to conjugated bile salts was grossly abnormal; this disturbance was somewhat less marked in the efferent jejunum and considerably less in the intraluminal contents of the afferent jejunum. An irregularly distributed lesion, consisting of swelling and vacuolation of microvilli and intracellular organelles, was demonstrated in the small-intestinal epithelium of blind-loop animals.

Impaired absorption of monosaccharides is a further consequence of bacterial contamination of the upper gut. It is suggested that this defect is caused by the presence of high levels of deconjugated bile salts produced by an abnormal anaerobic bacterial flora in the small intestine.

The syndrome of temporary monosaccharide malabsorption in infancy was first documented only a few years ago (Burke and Danks, 1966) and has subsequently been reported from various parts of the world (Zetterström and Waldenström, 1968; Harries and Francis, 1968; Wharton, Howells, and Phillips, 1968; Coello-Ramírez, Gutierrez-Topete, and Lifshitz, 1970; Lifshitz, Coello-Ramírez, and Gutierrez-Topete, 1970a and b). The aetiology of this disorder, in which there is a temporary inability to absorb all dietary monosaccharides in the first weeks or months of life is not known. However, a similar form of transient malabsorption of simple dietary sugars occurs in some infants after small-intestinal surgery in the first month of life when the absorptive defect is associated with a luxuriant overgrowth of faecal organisms in the upper small intestine (Burke and Anderson, 1966). Two recent reports indicate that similar bacteriological changes occur in patients not previously subjected to surgery (Gracey, Burke, and Anderson, 1969; Coello-Ramírez et al, 1970). The earlier of these reports showed the bacteriological abnormality to coincide with deconjugation of intraluminal bile salts; the authors suggested that this was due to the presence of an undetected abnormal anaerobic flora because of the almost exclusive ability of these organisms to deconjugate bile salts (Hill and Drasar, 1968). They further suggested that bacterial deconjugation of bile salts was closely related to the development of the monosaccharide transport defect.

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The present study examines the hypothesis that bacterial overgrowth and subsequent deconjugation of bile salts in the upper small intestine results in impaired monosaccharide absorption in rats with a jejunal blind loop \textit{in vitro} and \textit{in vivo}. Studies of the morphology of the small intestine in these animals were also performed.

Two substrates were chosen for these experiments. The first, arbutin (p-hydroxyphenyl-\beta-glucoside) is a synthetic analogue of D-glucose and is actively transported by the small intestine of the rat in the same way as glucose. Since it is not metabolized (Alvarado and Crane, 1964) it is convenient, appropriate, and recognized material for the study of the intestinal active sugar transport process (Gracey, Burke, and Oshin, 1971a). The other, D-fructose, was included because of its involvement in the clinical syndrome of temporary monosaccharide malabsorption and its probable normal passage across the small intestine by a carrier mechanism separate from that for D-glucose and other actively transported sugars.

\section*{Materials and Methods}

The experimental model used was the rat with a self-filling jejunal blind loop. Litter mates were used as controls. The blind loops were made surgically, then after two to three months the animals were studied by estimating faecal fat excretion over a period of five days; direct sampling and then examination of the aerobic and anaerobic bacterial flora of the jejunum (including the blind loop); ascertaining the pattern of conjugation of intraluminal jejunal bile salts by thin-layer chromatography; light and electron microscopy of the small intestine; and studying the uptake \textit{in vitro} and absorption \textit{in vivo} by the small intestine of a non-metabolized analogue of D-glucose and D-fructose.

The significance of these results was assessed by performing control studies in normal animals and comparing the results by standard statistical methods.

\section*{Animals}

Adult Wistar rats weighing 150-300 g were used throughout. The operative procedure was done under light open-ether anaesthesia using the technique of Cameron, Watson, and Witts (1949) which creates a self-filling jejunal blind loop about 8 cm long and maintains intestinal continuity through a side-to-end anastomosis.

\section*{Faecal Fat Excretion}

Stool collections were made while animals were individually housed in cages with raised floors of wide-mesh wire netting to prevent coprophagy. In the operated animals this was done two months after operation. Stool fat excretion was estimated by the method of van de Kamer, ten Bokkel, Huinink, and Weyers (1949). The same animals were used for faecal fat excretion studies and studies of intestinal sugar transport.

\section*{Bacteriological Methods}

Samples were taken from the upper third of the jejunum of normal animals and in the blind-loop animals either by aspirating directly or syringing and then aspirating the area studied with sterile buffer while the animal was lightly anaesthetized or by draining the appropriate area immediately after sacrificing the animal. Appropriate correction was made for the dilution factor involved. In the case of the blind-loop rats, specimens were obtained from the blind loop itself and from the 10 cm of jejunum immediately proximal to (ie, afferent) or distal from (ie, efferent) the junction with the blind loop. Specimens were either cultured within two hours of collection or deep-frozen at \(-20^\circ\) to \(-60^\circ\) C in 2 ml of transport medium (1.8 ml of glucose broth and 0.2 ml of glycerol) until cultured within one month of collection. Serial dilution was done before plating on horse blood agar (routine and anoxic), MacConkey agar, Sabouraud dextrose agar, and the medium of de Man, Rogosa, and Sharpe (1960). Anaerobic blood agar plates were incubated under strict anaerobic conditions at 37°C for up to six days. Organisms isolated on anaerobic plates were subcultured onto aerobic blood agar plates to exclude facultative anaerobes. Results are expressed as the log10 of the mean viable counts per millilitre of specimen.

\section*{Bile Salt Assays}

Specimens for bile salt assays were taken in the same way as those for bacteriological studies. In the normal animals specimens were taken from the upper, middle, and lower thirds of the jejunum; in the blind-loop animals from the same areas as the bacteriological specimens.

Bile salts were assayed using the sulphuric acid method (Sjövall, 1959) as described by Poley, Dower, Owen, and Stickler (1964) and modified by the use of thin-layer chromatography instead of paper chromatography. The solvent system, chloroform; methanol; acetic acid (80:12:3 by volume), was used and gave satisfactory separation of the principal conjugated bile salts, taurocholate and glycocholate, from the unconjugated salts, cholate and deoxycholate. Standard bile salts were obtained from Maybridge Chemical Company, Cornwall. Standards of taurocholate, glycocholate, deoxy-
Bacteria, bile salts, and intestinal monosaccharide malabsorption 685

cholate, and cholate were run on each thin-layer plate and the percentage recovery of these was determined. Recovery rates ranged from 70 to 75% for individual samples. The figures used in the correction, before expressing the results from each sample, were determined by the degrees of recovery of the internal standards used.

Taurocholate and glycocholate were assayed jointly but cholate and deoxycholate were each determined separately. Because of the inaccuracy of the method we did not attempt to determine concentrations of bile salts present in smaller amounts, such as chenodeoxycholate and its conjugates. To facilitate comparison between different animals and different areas of intestine, results are also expressed as a ratio of cholate and deoxycholate to taurocholate and glycocholate.

LIGHT AND ELECTRON MICROSCOPY
Specimens for light microscopy were fixed in formal saline and carefully orientated before mounting and staining with haematoxylin and eosin.

In three rats 1 mm cubes of tissue were fixed in 5% glutaraldehyde for 90 minutes, then transferred to sodium cacodylate-sucrose buffer for two days and postfixed in 2% osmium tetroxide buffered with veronal acetate. Specimens were then processed through dehydration in graded ethanol and embedding in Epon. Using a Reichert OMT2 ultratome, sections 1 μ thick were cut from blocks for identification and stained with toluidine blue. The blocks were trimmed appropriately, ultra-thin sections obtained and mounted on uncoated grids. They were stained with uranyl acetate and lead citrate and viewed in an AEI EM 6B electron microscope.

UPTAKE AND PERFUSION STUDIES
The uptake studies in vitro were done using the method described by Semenza and Mühlau (1969) which measures the uptake of substrate by everted pieces of tissue secured in a plexiglass tissue-holding apparatus.

For the perfusion studies in vivo the animals were lightly anaesthetized throughout the procedure with open ether. In normal animals a segment of mid-jejunum of 20 cm was used; in blind loop animals the afferent and efferent jejunum immediately proximal to and distal from the junction with the blind loop were used respectively. Particular care was taken to maintain the vascular integrity of the segment when preparing for perfusion. After ligating the common bile duct proximal and distal polyethylene cannulae (external diameter 3:5 mm internal diameter 2:0 mm) were introduced into the lumen of the gut through transverse incisions in its wall and secured by black silk ligatures so that a segment of approximately 20 cm in length could be perfused with a constant-rate perfusion apparatus at 10 ml per hour in a peristaltic direction. The cannulae were exteriorized, the perfused segment was returned to the abdominal cavity, and the abdominal wall closed by metal clamps during the perfusion. The first 30 minutes of the perfusion were used for equilibration conditions to be achieved; during the second 30 minutes the perfusate was collected continuously. At the end of the experiment the perfused segment was removed and its length measured, always by one of us (M.G.), by suspending the tissue lengthwise with a constant weight (25g) attached to the lower end. For technical and anatomical reasons it is not possible to study absorption by the blind loop itself in vivo.

SOLUTIONS USED FOR INCUBATIONS AND PERFUSIONS
The incubation media and perfusates were based on Krebs-Henseleit (1932) bicarbonate buffer at 37°C and pH 7.4, gassed for 60 minutes with 95% O₂ and 5% CO₂ immediately before the experiments and gassed throughout the incubations with the same gaseous mixture. For incubations in vitro, 3 mM arbutin (parahydroxyphenyl-β-glucoside) or 1 mM D-fructose was added to the solutions; at these concentrations accumulation against a concentration gradient has been demonstrated under similar conditions in vitro (Gracey, Burke, and Oshin, 1970 and 1971a). For the perfusions in vivo, the substrate concentrations were 10 mM arbutin and 5 mM D-fructose; at such concentrations significant inhibition of substrate absorption in vivo should be detectable by the analytical methods to be outlined.

Solutions containing D-fructose had 0.067 μCi/ml of 14C-D-fructose added as marker. Solutions for incubations in vitro with D-fructose also had 0.002 μmoles/ml of 3H-mannitol added to estimate the amount of sugar entering the tissue by passive diffusion (Bihler and Crane, 1962); this index was subtracted from the fructose uptake figures. A similar correction factor was applied to the arbutin uptake figures by estimating the extent of passive diffusion of 2-deoxy-D-glucose included in the incubation medium at a concentration of 1.5 mM.

After incubation in D-fructose the tissues were homogenized in distilled water at 100°C for five minutes and the extracts subjected to liquid scintillation counting. Possible conversion of D-fructose to D-glucose or lactate during incubation or perfusion was checked in the extracts and perfusates respectively on thin-layer chromatography using the solvent system, n-propanol: acetic acid: water (14:1:1 by volume), and estimating the degree of
recovery of fructose in relation to the total counts obtained from all these areas then examined separately by liquid scintillation counting. From the extracts following incubations in vitro 80% of radioactivity was recovered as D-fructose, the remainder being transferred to lactate. In the perfusates, following perfusion in vivo, 97% of total radioactivity was associated with D-fructose. Appropriate corrections for these figures were made when expressing the results. Polyethylene glycol 4000 was used in all perfusion experiments as a non-absorbable marker and corrections for changes in volume were made when calculating the results. Results are expressed as μmoles of substrate accumulated per millilitre of tissue water for the experiments in vitro, assuming a tissue water content of 80% (Crane and Mandelstam, 1960). For the perfusion experiments the results are expressed as μmoles of substrate transported per centimetre of intestine per hour.

CALCULATION OF DATA
Standard arithmetical methods were used throughout in achieving means and standard deviations (SD). Student's t test was used to obtain levels of statistical significance, and P values of <0.05 are taken as being significant.

ANALYTICAL METHODS
Arbutin, from Sigma Chemical Co, St Louis, USA, was determined as free phenol (Lowery, Rosebrough, Farr, and Randall, 1951; Folin and Ciocalteu, 1959); polyethylene glycol (Hopkin and Williams, Chadwell Heath) by a micro-modification of Hydén's method (1956); 2-deoxy-D-glucose (Sigma Chemical Company, St Louis, USA) was determined by the formation of malonic dialdehyde with periodate and on its condensation with barbituric acid (Waravdekar and Saslaw, 1957).

Radioisotopes were obtained from the Radiochemical Centre, Amersham. The radiochemical purity of 14C-D-fructose was 99% and of 3H-mannitol was 99%. Efficiency of liquid scintillation counting (Tracerlab CM-100 Spectrometer, London) was 85% for 14C and 50% for 3H.

Acids, solvents, and other chemicals were obtained from British Drug Houses, Poole.

Results

STOOL FAT EXCRETION
The mean daily stool fat excretion in normal animals (n = 24) was 194 (± 1 SD, 71) mg (range = 104-272 mg). The operated animals (n = 15) developed steatorrhoea, with a mean daily faecal fat excretion of 540 (± 1 SD, 160) mg (P < 0.001, range = 320-840 mg).

Fig. 1  Uptake of arbutin by small intestine of normal and blind-loop rats in vitro following incubation in a medium containing 3 mM arbutin (● = normal, n=8; ▲ = afferent jejunum of blind-loop rats, n=5; ■ = efferent jejunum of blind-loop rats, n=5; ○ = blind-loops, n=5). Results show mean uptakes and one standard deviation. Statistical comparison with normal uptake is indicated by asterisks; * indicates P < 0.05, ** indicates P < 0.01, and *** indicates P < 0.001.

SUGAR UPTAKE IN VITRO
Using 3 mM arbutin as substrate the mean uptake in normal animals was 2·6 (± 1 SD, 0·5) μmoles/ml tissue water at 10 minutes and at 30 minutes was 4·7 (± 1 SD, 0·8). The rate of uptake was less in all segments studied from the blind-loop animals, and after 30 minutes the mean uptake was significantly less than the control values. The results after this time were for afferent gut 2·4 (± 1 SD, 0·5, P < 0·001), blind-loop, 1·2 (± 1 SD, 0·6, P < 0·001), and for efferent gut 1·9 (± 1 SD, 0·6, P < 0·001) (Fig. 1).

With 1mM D-fructose as substrate the mean uptake in normal animals was 0·5 (± 1 SD, 0·2) μmoles/ml tissue water at 15 minutes, 0·9 (± 1 SD, 0·2) at 30 minutes, 1·3 (± 1 SD, 0·3) at 45 minutes, and 1·4 (± 1 SD, 0·3) at 60 minutes. The rate of uptake of D-fructose was less in all three areas from the blind-loop animals. After incubation for 30 minutes and longer all these results were significantly less than normal (Fig. 2).

SUGAR TRANSPORT IN VIVO
Using 10 mM arbutin as substrate, the jejunum of
Bacteria, bile salts, and intestinal monosaccharide malabsorption

normal animals transported 2.02 (± 1 SD, 0.27) μmoles/cm/hour. Less was transported by the blind loop animals; in the afferent jejunum the decrease was not significant but in the efferent jejunum the result was highly significant (Table I).

With 5 mM D-fructose as substrate the mean normal rate of intestinal transport was 0.016 μmoles/cm/hour. The rate of transport was less in both the afferent and efferent jejunum from the blind-loop rats. However, because of the small number of observations the statistical significance of these differences cannot be assessed.

Bacterial Flora

These results are shown in detail in Table II. In normal rats the aerobic population was less than 10⁶ viable organisms per ml and the anaerobic population was less than 10⁴ per ml. In all three areas from blind-loop rats there was an increase in both aerobes and anaerobes; the flora was most luxuriant in specimens from the blind loops. The changes were least marked in the afferent jejunum.

Bile Salts

The quantitative bile salt levels are given in Table III. This shows a marked difference in the amounts of conjugated and unconjugated bile salts from jejunal contents of normal and blind-loop animals. In all three areas studied in blind-loop rats there was a significant decrease in the levels of conjugated bile salts and a corresponding increase in the levels of deconjugated bile salts. If expressed as a ratio of free (unconjugated) to conjugated bile salts the following patterns emerge: in normal rats this ratio is less than 1; in specimens taken from all areas of blind-loop rats this ratio was exceeded. The free/conjugated ratios were highest in the blind loop (6:1) and more than 4:1 in the efferent jejunum.

Small Intestinal Morphology

Light microscopy of the three areas from the blind-loop animals showed no apparent morphological disturbances of the epithelial surface. There was a

<table>
<thead>
<tr>
<th>Substrate Transport (μmoles/cm/hour)</th>
<th>Number</th>
<th>Arbutin</th>
<th>P</th>
<th>Number</th>
<th>D-Fructose (mean)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>17</td>
<td>2.02 ± 0.27</td>
<td></td>
<td>6</td>
<td>0.016</td>
<td>0.011-0.025</td>
</tr>
<tr>
<td>Blind-loop rats</td>
<td></td>
<td></td>
<td></td>
<td>Afferent jejunum</td>
<td>5</td>
<td>1.51 ± 0.58</td>
</tr>
<tr>
<td>Efferent jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.21 ± 0.36</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table I Intestinal sugar transport by normal and blind-loop rats in vivo

1Perfusate contained 10 mM arbutin or 5 mM D-fructose respectively.
greater than normal degree of cellular infiltration of the lamina propria, predominantly by plasma cells and lymphocytes.

Electron-microscopic examination revealed an irregularly distributed pattern of ultramicroscopic changes in the epithelium of the blind loop and distal gut areas. This change, shown in Figs. 3 and 4, demonstrates ‘blistering’ or ‘ballooning’ of the microvilli associated with underlying mitochondrial swelling in the affected cells. A characteristic feature of these lesions was their apparent confinement to isolated cells which often adjoined cells of normal appearance.

Discussion

The studies reported here were prompted by recent clinical experience with young infants with the syndrome of temporary monosaccharide malabsorption, where an abnormal small-intestinal bacterial flora and bile salt deconjugation are associated with a temporary inability to absorb all dietary monosaccharides (Gracey et al., 1969). In the present experiments impaired intestinal transport of two monosaccharides has been demonstrated in vitro and in vivo in rats with a surgically constructed, self-filling jejunal blind loop. One of the substrates used (arbutin) is normally carried by the established intestinal active sugar transport pathway (Alvarado and Crane, 1964): the other, D-fructose, is probably carried by a different mechanism. Along with this impairment of carbohydrate absorption were found a grossly abnormal aerobic and anaerobic small-intestinal bacterial flora, extensive deconjugation of intraluminal bile salts, and ultrastructural changes in the epithelial cells of the small intestine.

Intestinal sugar transport was markedly impaired in vitro in tissue from the blind loop and the intestine immediately distal to it. This inhibitory effect was present, but less marked, in jejunal proximal to the blind loop. A previous study has shown everted sacs of small intestine of blind-loop rats have impaired absorption of glucose in vitro (Baraona, Palma, Navia, Salinas, Orrego, and Espinoza, 1968), but direct evidence of impaired carbohydrate absorption has not previously been found in vivo. That impaired intestinal sugar transport occurs in blind-loop rats in vivo was clearly shown in the
Bacteria, bile salts, and intestinal monosaccharide malabsorption

Fig. 3 Electron micrograph of small-intestinal epithelial surface from a blind-loop animal illustrating two normal epithelial cells with a goblet cell between them and an abnormal epithelial cell on the right. The latter has swollen, poorly staining mitochondria and cytoplasmic inclusions of less than normal density. The overlying microvilli are ‘ballooned’ and poorly staining. The microvilli overlying the normal cell on the left are cut tangentially and illustrate the differences in morphology and staining characteristics between these and the ‘ballooned’ microvilli overlying the abnormal cell (x 4000).

The present study where absorption of arbutin was significantly depressed in small intestine distal to the blind loop. Although the numbers of experiments with D-fructose are too small to make statistically valid comment, there was a similar trend shown with this substrate. These findings suggest that disturbance of the intestinal active sugar transport pathway and that involved in the transport of D-fructose occurs as a consequence of bacterial contamination of the upper gut.

The pathogenesis of disturbed carbohydrate absorption in this situation is not clear. Blind-loop rats have previously been shown to have impaired urinary excretion of D-xylose (Donaldson, 1967) but the degree of intraluminal consumption of the sugar by bacteria was unknown and cast doubts on the pathogenesis of the absorptive defect. This proposed mechanism of apparently artefactual disappearance of the sugar has since been supported by others (Goldstein, Karacadag, Wirts, and Kowlessar, 1970). However, its clinical significance, in quantitative terms, remains doubtful and evidence against the importance of bacterial utilization in vivo comes from acute experiments in blind-loop rats in which previous clearing of the gut lumen of bacteria does not reverse the D-xylose absorptive defect (R. M. Donaldson Jr, 1970, personal communication).

How might impaired monosaccharide absorption develop in this situation? The present data show close correlation of the severity of the absorptive defect with the degree of abnormality of the intestinal bacterial flora and the extent of deconjugation of bile salts, which was most marked in the contents of the blind loop and less so, but still very considerable, in the contents of the efferent jejunum. There is now considerable evidence available which suggests that
disturbance of bile salt conjugation might be responsible for impaired transport of sugar in these animals. Several groups of workers have shown that unconjugated bile salts impair the intestinal transport of sugars in vitro (Forth, Rummel, and Glasner, 1966; Pope, Parkinson, and Olson, 1966; Gracey et al, 1971a). In a previous study in blind-loop rats (Baraona et al, 1968) and in the present one deconjugated bile salts were found in intestinal contents in association with impaired carbohydrate absorption in vitro. The recent demonstration that the inhibitory effect of these substances is acutely
Bacteria, bile salts, and intestinal monosaccharide malabsorption

reversible (Gracey et al, 1971a) in a newly described system in vitro (Semenza and Mühlaupt, 1969) overcomes the earlier criticism that the inhibitory effect demonstrated in vitro might simply be due to irreversible tissue damage (Dietschy, 1967) and points to the probable relevance of this effect in situations in vivo. The importance of this effect in vivo has recently been confirmed by other experiments from this laboratory where perfusions of the unconjugated bile salts, cholate and deoxycholate, in normal rats have been clearly shown to have an inhibitory effect on intestinal sugar absorption in vivo (Gracey, Burke, and Oshin, 1971b). It is important to recall that the ability of intestinal bacteria to deconjugate bile salts is almost entirely confined to anaerobes (Hill and Drasar, 1968). It seems most likely, then, that in the present studies the large numbers of anaerobic bacteria found within the intestinal lumen and possessing this ability, mainly Bacteroides sp. and Bifidobacteria, have led to deconjugation of bile salts which resulted in impaired intestinal transport of monosaccharides.

What is the significance of the ultrastructural changes found in the microvilli and mitochondria of the small intestine of these blind-loop animals? Remarkably similar morphological changes have been demonstrated by Shiner (1969) by instilling deconjugated bile salt into the upper gut of normal rats in vivo and subsequently examining the intestine by electron microscopy. Although the present electron-microscope studies are of limited extent they indicate that ultrastructural changes may occur together with bacterial contamination of the upper small intestine. This view is at variance with the reported findings of previous light- (Donaldson, 1965) and electron-microscope studies (Kjaerheim and Nygaard, 1968), although Paulley (1969) has presented evidence of morphological damage to brush borders of villous tips in patients and experimental animals with bacterial overgrowth in the small intestine. Clearly, more thorough examination of this question is needed in future studies.

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