

## Effects of olsalazine in the jejunum of the rat

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**SUMMARY** Olsalazine (ADS) is the azo-linked dimer of 5-aminosalicylic acid (5-ASA). It is of value for the management of patients with ulcerative colitis but may be associated with increasing diarrhoea in a few. This study examines the effect of 5-ASA and ADS on small intestinal transport systems of the rat. Krebs-Ringer-bicarbonate solution was circulated through the lumen of a jejunal segment and the appearance of fluid, glucose and lactate on the serosal surface was shown to be linear over a two hour period. Addition of 5-ASA (10 mmol/l) or ADS (5 mmol/l and 10 mmol/l) caused a significant inhibition both of fluid transport ( $p < 0.001$ ), and of the appearance of glucose ( $p < 0.001$ ) and lactate ( $p < 0.001$  for 5 mmol/l and 10 mmol/l ADS,  $p < 0.01$  for 10 mmol/l 5-ASA). The uptake of glucose by rings of rat jejunum was shown to be markedly reduced by ADS. Experiments substituting glucose with either sucrose or 2-aminoisobutyric acid showed that ADS (5 mmol/l, 10 mmol/l) also inhibited the serosal appearance of fructose and the amino acid. These results show that 5-ASA and ADS, at concentrations which could be expected in the jejunum of patients receiving therapeutic doses, are able to inhibit small intestinal transport systems. The resulting increase in load on the diseased colon could be important for the pathogenesis of diarrhoea.

Sulphasalazine has been the main therapeutic agent for inflammatory bowel disease for the last 40 years after its introduction by Svartz.<sup>1</sup> It is now known that the 5-aminosalicylic acid moiety of sulphasalazine is the active agent in ulcerative colitis and that the sulphapyridine portion acts only as a carrier, the azo bond being split in the lumen of the colon by the bacterial enzyme azo-reductase.<sup>2,3</sup> Most of the adverse effects of the drug are related to the sulphapyridine moiety.<sup>4</sup> For maintenance therapy it was found that the larger the dose of sulphasalazine, the lower the relapse rate, but with increased side effects of the drug.<sup>5</sup> Attempts to discover a form of molecule which could carry the maximum therapeutic amount of the 5-aminosalicylic moiety to its site of action without being degraded or absorbed in the upper part of the intestine led to the suggestion that azodisalicylic acid (ADS, Olsalazine) could be a promising candidate. In this compound two molecules of 5-aminosalicylic acid (5-ASA) are bound by an azo

bond which is split in the colon by an azo-reductase. Willoughby *et al*<sup>6</sup> found that there were no substantial side effects when ADS was given orally in healthy volunteers. In one trial of ADS in patients with ulcerative colitis, however, a substantial number complained of loose stools and increased frequency of bowel movement.<sup>7</sup>

The diarrhoea that accompanies ADS treatment could be because of an abnormality in water and electrolyte transport in the small intestine leading to an increased fluid load to the colon. The present study investigates whether there is any abnormality in fluid transport in the jejunum when it is exposed to ADS. In addition carbohydrate transport and metabolism, and amino acid transport have been examined. The metabolism of ADS itself by the jejunum epithelium has also been studied.

### Methods

#### ANIMALS

Male Wistar rats (210-280 g) supplied by the Local Laboratory Stock (MRC Lab) were maintained on a

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standard synthetic diet and kept in constant conditions of temperature and light. Experiments were done between 1030 and 1130 am on animals deprived of food but given 5% glucose water to drink overnight.

#### APPARATUS

Krebs-Ringer-bicarbonate solution was circulated through the lumen of a segment of jejunum *in vitro* as described by Parsons and Shaw.<sup>8</sup> The cannulated segment was suspended in 150 ml liquid paraffin (sg 0.850–0.865) contained in a water jacketed (38°C) vessel, and the secreted serosal fluid was collected from the bottom of the vessel. Fluid (total volume 150 ml) circulated through the lumen of the intestinal segment by means of a peristaltic pump (Gilson minipuls HP<sub>4</sub>) at a constant rate of 26 ml/min and the circulation fluid, kept at 38°C, was constantly gassed with a mixture of 95% O<sub>2</sub>-5% CO<sub>2</sub>. Large bubbles of gas passing through the intestinal segment stirred the luminal contents.

#### CIRCULATION AND INCUBATION FLUIDS

The composition of the Krebs-Ringer-bicarbonate solution was as follows (mM): NaCl, 120; KCl, 4.5; MgSO<sub>4</sub>, 1.0; Na<sub>2</sub>HPO<sub>4</sub>, 1.85; NaH<sub>2</sub>PO<sub>4</sub>, 0.2; NaHCO<sub>3</sub>, 25; CaCl<sub>2</sub>, 1.25. The fluid was gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> (v/v) at least half-an-hour before adding the CaCl<sub>2</sub>.

Twenty eight mmol/l glucose was added to the solution in all circulation experiments except when 28 mmol/l sucrose was used instead of glucose.

In some experiments 10 mmol/l 2-aminoisobutyric acid (AIB) and C<sup>14</sup> 2-aminoisobutyric acid (final concentration 2.5 µCi C<sup>14</sup> 2-aminoisobutyric acid in 150 ml of fluid) were added to the glucose-Ringer circulation fluid. 2-Aminoisobutyric acid is an amino acid analogue which is transported as natural amino acid into cells but which cannot be metabolised.<sup>9,10</sup>

#### PREPARATION OF THE INTESTINE

##### (a) Circulation experiments

Rats were anaesthetised with sodium pentobarbitone (Nembutal, Abbot) given subcutaneously in a dose of 70 mg/kg body weight. The abdomen was opened by a longitudinal incision and the jejunum, a few centimetres beyond the ligament of Treitz, was ligated, then cut and cannulated beyond the ligature. Fifteen to 20 cm of jejunum were selected and the distal end ligated and cannulated. The segment was flushed to wash out the debris for 45–60 seconds with Krebs-Ringer bicarbonate solution (without glucose) maintained at a constant temperature of 38°C and gassed continuously with the O<sub>2</sub>/CO<sub>2</sub> mixture. A ligature around the mesenteric blood vessels supplying the segment was tied and the jejunal segment was

cut carefully from the mesentery as near as possible to the intestine. The segment was then wiped with tissue to remove blood and fluid; the cannulae were connected to the main apparatus and the segment immersed in the liquid paraffin; the pumping of glucose (28 mmol/l)-Ringer was started and timing begun.

Fluid started to appear as a sweat on the serosal surface about five minutes after starting the circulation. At 15 minutes the sample of serosal fluid at the bottom of the vessel was collected and discarded. The first sample was taken at 20 minutes, and thereafter 10 samples were collected at 10 minute intervals up to 120 minutes in small graduated test tubes. After centrifuging, the volumes were measured and then 100 µl samples were taken out and added to 2 ml of ice cold, 6% perchloric acid. After neutralisation the samples were assayed for glucose and lactate.

Some samples of serosal fluid were kept frozen at –20°C for high performance liquid chromatography (HPLC) assay of drug metabolites.

At the end of the experiment the intestine was removed from the apparatus, cut open along the mesenteric border and blotted carefully. The wet and, after drying in a hot air oven for 24 hours at 60°C, the dry weights were measured.

#### DRUGS

At 50–55 minutes in circulation experiments about 10 ml circulation fluid were taken out with a syringe from the reservoir and a solution of ADS was made so that the final concentration in the fluid would become 1 mmol/l, 5 mmol/l or 10 mmol/l. It was then added to the circulation apparatus at 60 minutes. In the same way a 10 mmol/l solution of 5-aminosalicylic acid was prepared, this needing a few drops of NaHCO<sub>3</sub> (1M) to dissolve it.

##### (b) Ring experiments

Male Wistar rats were anaesthetised and dissected in a similar way and a proximal segment of jejunum was removed after injecting 20–30 ml of ice cold saline (9 g NaCl/l) through the lumen to clean out the debris. Rings of 1–2 mm in width were cut; they were randomised, blotted and weighed in batches of 10. One batch was put in a hot air oven for 24 hours to measure the dry weight. The remaining batches were incubated in 10 ml of Krebs-Ringer-bicarbonate solution containing 10 mmol/l glucose, for 10 minutes in a water bath at 38°C with constant shaking at a rate of 100/min. Each incubating flask was well gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> and then sealed off with an airtight rubber bung before placing in the water bath. In each experiment batches were incubated with 0, 1 mmol/l, 5 mmol/l, and 10 mmol/l ADS added to the Krebs-Ringer-bicarbonate-glucose solution. After

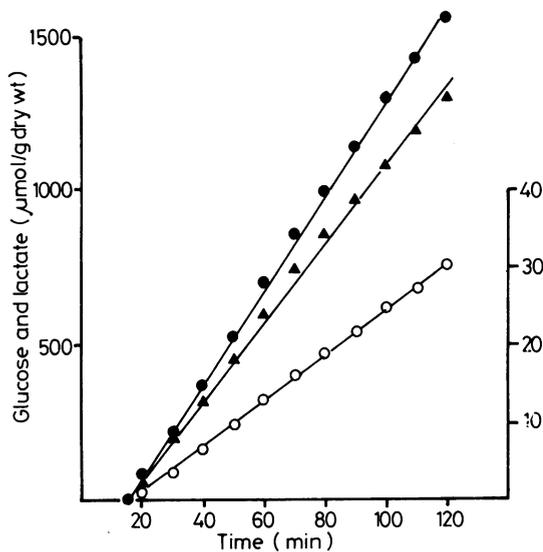


Fig. 1a Glucose (●) and lactate (▲) accumulating in the serosal fluid (○) secreted from a recirculated segment of rat jejunum in vitro. Recirculation fluid was Krebs-Ringer bicarbonate buffer containing 28 mmol/l glucose.

10 minutes a 100 µl sample was taken immediately from the incubation fluid for estimation of glucose, and 10 mmol/l of ice cold, 6% perchloric acid was added to the rest of the incubating solution. After a few minutes of vigorous shaking a sample was taken from the supernatant for estimation of lactate.

#### BIOCHEMICAL ANALYSIS

Enzymatic assays were done for glucose, lactate, and fructose (Bergmeyer, 1974, 2nd English Edition) and the results were expressed in µmol/g dry weight of intestine. Rates of appearance of serosal fluid, glucose and lactate before and after addition of ADS were calculated from the accumulated data from all circulation experiments in a particular group by linear regressions relating appearance with time; the rates were expressed as µmol/g dry weight/minute ± Standard error of the mean (SEM). Tests of significance were done by comparing the relevant regression coefficients using the appropriate t-values.

Radioactivity of  $C^{14}$  2-aminoisobutyric acid was counted in a liquid scintillation counter (LKB, 215 Rackbeta) after adding 100 µl of serosal fluid to 10 ml of optiphase (LKB).

#### HPLC ASSAY

5-ASA and n-Ac-5-ASA were determined by an HPLC method (Personal communication; N-O Ahnfelt, 1984). Twenty microlitre samples were separated isocratically at 1 ml/min on an octadecyl-

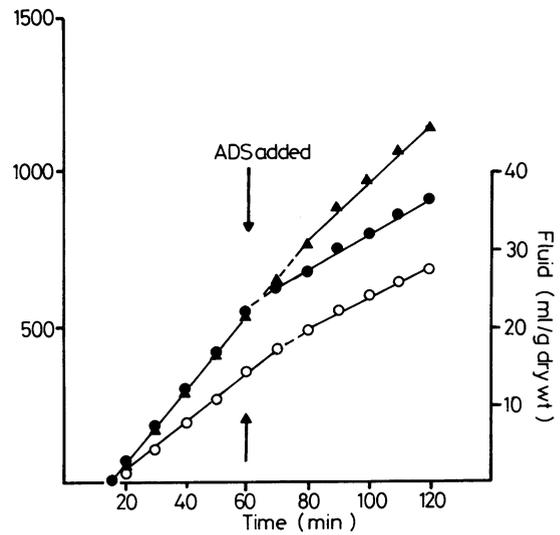


Fig. 1b As for (a) with 10 mmol/l ADS added to the recirculation fluid at 60 minutes.

silica reverse-phase column (Merck LiChro CART system, 250×4 mm RP-18 column with 4×4 mm precolumn; BDH Chemicals, Poole BH12 4NN, UK) using fluorescence detection (excitation 312 nm, emission 469 nm; Shimadzu RF-530, Dyson Instruments, Hetton DH5 OAT, UK). The eluant was 0.01 M tetrabutylammonium sulphate, 0.0044 M  $Na_2HPO_4$ , adjusted to pH 7.4, mixed with methanol 71:29 (v/v). Solvents and samples were filtered (0.45 µm).

## Results

#### CIRCULATION EXPERIMENTS

That the appearance of fluid, glucose and lactate on the serosal side of the intestinal segment is linear over the time course of the circulation experiments is shown by a typical control experiment in Figure 1a where the accumulated products are plotted against

Table 1 Rates of serosal fluid appearance µl/g dry wt/min

	15-60 min n=36	80-120 min n=30	p value
Controls	281 ± 5	290 ± 5	NS
1 mmol/l ADS	304 ± 4	289 ± 8	NS
5 mmol/l ADS	303 ± 10	168 ± 6	<0.001
10 mmol/l ADS	328 ± 6	154 ± 14	<0.001
10 mmol/l ASA	355 ± 8	223 ± 10	<0.001

Medium contained 28 mmol/l glucose. Drug added at 60 min. n=6 animals×5 or 6 time points; values are means ± SEM. Values for p are given for comparisons between 80-120 min and 15-60 min. NS=not significant.

Table 2 Rates of appearance of glucose and lactate in serosal secretions  $\mu\text{mol/g dry wt/min}$ 

	15–60 min n=36		80–120 min n=30	
	Glucose	Lactate	Glucose	Lactate
Controls	14.5 $\pm$ 0.4	13.3 $\pm$ 0.7	14.1 $\pm$ 0.3	14.3 $\pm$ 1.0
1 mmol/l ADS	13.9 $\pm$ 0.6	9.86 $\pm$ 0.48	15.0 $\pm$ 0.7	10.7 $\pm$ 0.2
5 mmol/l ADS	15.7 $\pm$ 0.8	10.0 $\pm$ 0.5	10.2 $\ddagger$ $\pm$ 0.6	6.49 $\ddagger$ $\pm$ 0.32
10 mmol/l ADS	14.5 $\pm$ 0.4	11.4 $\pm$ 0.7	4.62 $\ddagger$ $\pm$ 0.47	6.96 $\ddagger$ $\pm$ 0.89
10 mmol/l 5-ASA	19.4 $\pm$ 0.6	13.2 $\pm$ 0.6	11.0 $\ddagger$ $\pm$ 0.4	10.3* $\pm$ 0.8

Medium contains 28 mmol/l glucose. Drug added at 60 min. n=6 animals $\times$ 5 or 6 time points; values are means $\pm$ SEM.

\*p<0.01  
 $\ddagger$ p<0.001 } comparing 80–120 min with 15–60 min.

time. The preparation clearly continues to secrete fluid, glucose and lactate at a constant rate, shown by the slope of the line, over two hours.

In experiments where drugs were tested, they were added at 60 minutes so that the first half of the experiment acted as a control for the second half. When 5 mmol/l or 10 mmol/l ADS or 10 mmol/l 5-ASA was added to the fluid circulating through the lumen of the intestine there was a marked change in the rate of appearance of serosal products (Fig. 1b).

Table 1 shows the rate of serosal fluid appearance, and Table 2 shows the rate of glucose and lactate appearance in control experiments, and in those with 10 mmol/l 5-ASA, and 1, 5, and 10 mmol/l ADS added at 60 minutes. The rates between the first and second halves of the experiment are significantly different (p<0.001) when the drugs are used at 5 or 10 mmol/l concentrations. There was no significant difference when 1 mmol/l ADS was added.

In control experiments, the concentration of glucose in the serosal secretion was normally more than 50 mmol/l compared with 28 mmol/l initially in the lumen. With 10 mmol/l ADS added to the circulation fluid, serosal glucose concentration fell to below 30 mmol/l. The concentration of lactate in the serosal secretion, about 40 mmol/l in controls, did not fall with 10 mmol/l ADS but was slightly raised.

When lactate production is expressed as a fraction

Table 3 Uptake of glucose and production of lactate by rings of rat jejunum

	Uptake of glucose $\mu\text{mol/g dry wt/min}$	Appearance of lactate $\mu\text{mol/g dry wt/min}$	
		+glucose	-glucose*
Controls	44.0 $\pm$ 2.6	99.0 $\pm$ 3.6	4.1
1 mmol/l ADS	48.0 $\pm$ 3.1	95.3 $\pm$ 5.2	14.1
5 mmol/l ADS	30.8 $\pm$ 3.1	68.2 $\pm$ 4.7	32.0
10 mmol/l ADS	6.25 $\pm$ 0.60	58.3 $\pm$ 4.4	55.2

Rings incubated in Ringer-carbonate solution $\pm$ 10 mmol/l glucose with different concentrations of ADS for 10 minutes. Values are means of 6 experiments $\pm$ SEM. \*2 experiments only.

of the total hexose appearance in the serosal fluid it increased from 0.28 before the addition of 10 mmol/l ADS to 0.43 after addition. With 10 mmol/l 5-ASA the increase was from 0.25 to 0.32, but there was no change in this fraction when 1 or 5 mmol/l ADS were used.

Histology of the jejunal segment at the end of the experiment showed the mucosa was intact and no significant changes were observed with or without ADS added to the circulation fluid. The tissue water in the segment did not change markedly with addition of the drugs.

#### RING EXPERIMENTS

The circulation experiments measure serosal products and give little information on uptake from the luminal fluids into the intestinal epithelium. In order to measure the utilisation of glucose, rings of jejunum were used and the uptake of glucose and the production of lactate were measured in the presence and absence of ADS (Table 3). It is seen that ADS markedly reduced the glucose uptake into the tissue. The lactate formed in the presence of high concentrations of ADS, however, greatly exceeded that expected from the glucose that was used. It is also seen that in the absence of glucose ADS induced lactate production from sources other than glucose.

#### SUCROSE

When the glucose in the circulation fluids was replaced by sucrose, fluid transport continued and glucose, fructose, and lactate appeared in the serosal secretions (Table 4); after the addition of 10 mmol/l ADS after 60 minutes, fluid transport, and the secretion of glucose, fructose and lactate at the serosal surface were greatly reduced. The fact that, even with 10 mmol/l ADS, fructose continued to appear means that ADS does not completely inhibit the hydrolysis of sucrose. The ratio of fructose to glucose rates of appearance in the serosal fluid was 0.24 with no drug and 0.34 with 10 mmol/l ADS. The fraction of total secreted hexose appearing as lactate

Table 4 Rates of fluid, glucose, fructose and lactate appearance in the serosal secretions. 28 mmol/l sucrose in luminal fluid

	Fluid µl/g dry wt/min	Glucose µmol/g dry wt/min	Fructose	Lactate
Controls				
15–60 min n=24	258±8	10.7 ±0.4	2.29±0.15	8.53 ±0.24
80–120 min n=20	281±4	11.8 ±0.7	2.68±0.19	9.95* ±0.31
n=4 animals×5 or 6 time points				
10 mmol/l ADS added at 60 min				
15–60 min n=36	285±8	11.4 ±0.4	2.77±0.21	9.77 ±0.48
80–120 min n=30	160±7	4.49* ±0.19	1.51±0.14	6.51* ±0.44
n=6 animals×5 or 6 time points				

Values are means±SEM. \*p<0.001 comparing 80–160 min with 15–60 min.

Table 5 Rates of appearance of fluid and 2-aminoisobutyric acid in serosal secretions

	n	Fluid µl/g dry wt/min		2-aminoisobutyric acid µmol/g dry wt/min	
		20–60 min	80–120 min	20–60 min	80–120 min
Controls	20	380±26	382 ±21	3.92±0.50	4.52 ±0.46
1 mmol/l ADS	20	342±22	321 ±14	2.76±0.46	3.23 ±0.40
5 mmol/l ADS	20	386±37	276* ±15	3.56±0.54	3.07 ±0.17
10 mmol/l ADS	30	423±32	212†±17	4.31±0.42	2.58†±0.19

Medium contains 28 mmol/l glucose. Drug added at 60 min. n=no of animals×5 time points. Values are means±SEM.

\*p<0.01  
†p<0.001 } comparing 80–120 min with 15–60 min.

increased after the addition of 10 mmol/l ADS from 0.26 to 0.35.

#### 2-AMINOISOBUTYRIC ACID (AIB)

Table 5 shows the effects of increasing concentrations of ADS on the transport of the amino acid AIB into serosal secretions. It will be seen that at concentrations of 5 and 10 mmol/l ADS the transport of fluid and of the amino acid was inhibited. The concentration of AIB in the serosal fluid increased steadily over 120 minutes reaching a maximum of 12 mmol/l from an initial concentration of 10 mmol/l in the lumen, and the presence of the drug did not affect this concentration.

#### HPLC RESULTS

High performance liquid chromatography allowed the acetylated form of 5-ASA to be monitored in the serosal fluid samples. The concentration increased with duration of the experiment with glucose plus 10 mmol/l ADS in the lumen. When AIB was present with glucose and the drug in the circulation fluid even higher concentrations of acetyl 5-aminosalicylic acid were obtained in the serosal fluid (Table 6).

#### Discussion

The principal findings in the circulation experiments

are that ADS has inhibitory effects on intestinal fluid transport. The effects are not marked at 1 mmol/l ADS but increase up to 10 mmol/l ADS – a concentration which is likely to be present *in vivo* in the patient receiving therapeutic doses. Because it is known that fluid transport is isotonic<sup>9</sup> this must mean that salt transport is also inhibited.

#### GLUCOSE AS SUBSTRATE

In ring experiments glucose utilisation was shown to be depressed and this could be caused either by the direct inhibitory effect on glucose transport into the enterocyte across the brush border, or by inhibition of glucose metabolism within the enterocyte or both. In any case the reduction of fluid transport found in

Table 6 Transport of acetyl 5-amino salicylic acid to serosal secretions

Circulation fluid	Experiments (n)	Times after adding ADS to the circulation fluid		
		20 min	40 min	60 min
28 mmol/l glucose with 10 mmol/l ADS	4	0.06±0.01	0.27±0.04	0.76±0.11
28 mmol/l glucose 10 mmol/l AIB with 10 mmol/l ADS	4	0.22±0.01	1.00±0.11	1.63±0.18

Values are means±SEM in nmol/g dry wt.

the presence of ADS (and 5-ASA) is associated with a reduction in glucose transport across the epithelium and in lactate appearance.

#### SUCROSE AS SUBSTRATE

In control circulation experiments it was found that sucrose was a satisfactory substrate for fluid transport, and that fructose as well as glucose and lactate was secreted into the serosal fluid.<sup>10</sup> In the presence of ADS (up to 10 mmol/l) fluid transport was again inhibited and the secretion of glucose, fructose and lactate also reduced. The fact that some fructose still appeared with 10 mmol/l ADS in the lumen indicates that the hydrolysis of sucrose at the brush border is not completely inhibited by the drug.

#### LACTATE PRODUCTION

In control circulation experiments the lactate, calculated as hexose, accounted for about 28% of carbohydrate in the serosal secretions. With 10 mmol/l ADS the fraction increased to 43%. From ring experiments, however, it was found that in the absence of glucose, both 5 and 10 mmol/l ADS produced significant amounts of lactate compared with controls. Thus there is little doubt that the drug has metabolic effects that include stimulation of lactate production from sources other than exogenous glucose. For example it is possible that in the ring experiments ADS stimulated the breakdown of glycogen in the smooth muscle fibres. With high concentrations of ADS in the circulation experiments disproportionately more lactate appeared in the serosal secretions which supports the view that ADS may cause the production of lactate from sources other than exogenous glucose.

Although ADS concentrations in the serosal secretions were not measured in these experiments, when ADS was present in the lumen the serosal secretions took on the same yellow coloured appearance as the lumen. Certainly the acetylated metabolite (acetyl 5-amino salicylic acid) appeared in the serosal secretions in increasing concentrations with time.

#### 2-AMINOISOBUTYRIC ACID

Transport of the non-metabolised amino acid analogue which in these experiments was not concentrated in the serosal secretions, was inhibited by ADS. The effects of the drug are not therefore confined to hexose substrates. Again, whether the effect is produced on the transport system of the brush border, or elsewhere in the epithelium can only be determined by other experiments.

#### CLINICAL IMPLICATIONS

The effects of ADS on small intestinal transport

were seen at 5 and 10 mmol/l concentrations, concentrations which could be expected in the jejunum of patients receiving therapeutic doses of 1 g bd. Hence, similar inhibition of transport mechanisms in man may occur *in vivo* and this is supported by observations in patients with an ileostomy. Sandberg-Gertzen *et al*<sup>13</sup> found that the mean increase in ileostomy effluent was 220 ml daily on a dose of 0.5 g bd of ADS, and 425 ml with a dose of 1 g bd. This represents increases of 43.5% and 84.1% respectively. For a healthy subject with an intact colon, such an increase in ileal outflow would not necessarily have an effect. The reserve capacity of a diseased colon with respect to salt and water absorption, however, is limited<sup>14</sup> and increasing ileal outflow would be a potent mechanism for diarrhoea. It is pertinent that the majority of patients with ulcerative colitis who have developed diarrhoea while receiving ADS were those who had an extensive colitis (Sandberg-Gertzen *et al* 1985).<sup>7</sup>

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