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Comparison of 5-aminosalicylic acid and N-acetylaminosalicylic acid uptake by the isolated human colonic epithelial cell

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Abstract

Isolated human colonic epithelial cell suspensions were incubated with either 0·1 mM 5-aminosalicylic acid (5-ASA) or 0·1 mM acetylaminosalicylic acid (Ac-ASA) for up to two hours. Intra- and extracellular 5-ASA and Ac-ASA were measured by high performance liquid chromatography. Mean 5-ASA uptake in one hour was 160·5 nmol/g dry weight, compared with an Ac-ASA uptake of only 5·75 nmol/g dry weight. No unchanged 5-ASA was detected inside the cell. Repeated washing had no effect on the intracellular Ac-ASA concentration. This discrepancy in drug uptake may explain why Ac-ASA seems to be ineffective when given to patients with ulcerative colitis.

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5-Aminosalicylic acid (5-ASA) is the active ingredient of sulphasalazine in ulcerative colitis,[1] and is thought to work by a local mechanism. The absorption of 5-ASA by the colon is poor, resulting in very low serum and urine concentrations[2] and high faecal concentrations.[3] The main metabolite of 5-ASA is N-acetyl ASA (Ac-ASA), and the drug is found predominantly in this form in the body. The site of acetylation of 5-ASA was widely assumed to be the liver, but it has recently been shown that the human colonic epithelial cell is capable of acetylating 5-ASA, and that N-acetyltransferase activity is present in the cytosol.[4] In addition, it has been shown that Ac-ASA is the form of the drug predominantly found in the rectal mucosa of patients taking oral sulphasalazine.[5]

It has been suggested that this acetylation process may inactivate the drug, and the results of clinical treatment trials of Ac-ASA have been conflicting,[6-8] with the drug being ineffective in two out of three trials. One possible explanation for this discrepancy might be that Ac-ASA is less well taken up by the colonic mucosa, and there is indirect evidence to suggest that this so.[9,10] The aim of this study was to compare the uptake of 5-ASA with that of Ac-ASA into the isolated human colonic epithelial cell.

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Methods

Fresh colonocyte suspensions were prepared according to the method of Roediger and Truelove.[11] Briefly, fresh surgical resection specimens were obtained from normal areas of colon at least 5 cm distant from a carcinoma. Mucosal strips were incubated in Ca^{++}/Mg^{++} free Krebs-Henseleit saline, pH 7·4, with 10 mM EDTA and 20 mM dithiothreitol, for 30 minutes at 37°C. Mechanical agitation released the epithelial cells into a suspension, which was then washed several times before use. This cell suspension was the basis for all the experiments. Cell viability was checked by both Trypan blue exclusion and parallel estimation of lactate production from 5 mM glucose. Lactate was measured in the incubation medium by a spectrophotometric assay in which lactate dehydrogenase (Boehringer – EC 1.1.1.27) catalysed the reduction of NAD to NADH, which was subsequently measured at 340 nm. Both 5-ASA and Ac-ASA were kindly supplied by Pharmacia AB, Uppsala, Sweden, and were prepared as neutral solutions.

INCUBATION WITH 5-ASA

Aliquots (2 ml) of cell suspension were incubated, in duplicate or triplicate, with 0·1 mM 5-ASA and 5 mM glucose for one hour at 37°C in a shaking water bath (at 70–80 oscillations/minute). The incubation was terminated by rapid centrifugation, following which the supernatant was analysed for the presence of Ac-ASA by high performance liquid chromatography (HPLC). Intracellular 5-ASA and Ac-ASA were measured by resuspending the cell pellet in 1 ml 0·05 M phosphate buffer, and disrupting it ultrasonically by six, 5 second bursts using an ultrasonicator (MSE). Control experiments were performed to assess the ability of the Krebs-Henseleit saline, and also the supernatant of a cell suspension, to acetylate 5-ASA. It has previously been shown that there is no acetyltransferase enzyme activity in the epithelial cell membrane.[4] The intracellular and extracellular production of Ac-ASA was therefore deemed to be the result of 5-ASA uptake and subsequent metabolism – that is, equivalent to 5-ASA uptake.

INCUBATION WITH AC-ASA

Aliquots (2 ml) of cell suspension were incubated, in duplicate or triplicate, with either 0·1 mM or 1·0 mM Ac-ASA and 5 mM glucose for one or two hours at 37°C in the shaking water bath. After incubation, the suspension was rapidly centrifuged and the supernatant removed. The cell pellets were washed three times in phosphate buffered saline (PBS) (2 ml) to remove any adherent drug before being resuspended in 1 ml 0·05 M phosphate buffer. This suspension was disrupted ultrasonically by six, 5 second bursts using an ultrasonicator (MSE), then centrifuged at high speed for five minutes, thus allowing the intracellular drug content to be measured. The

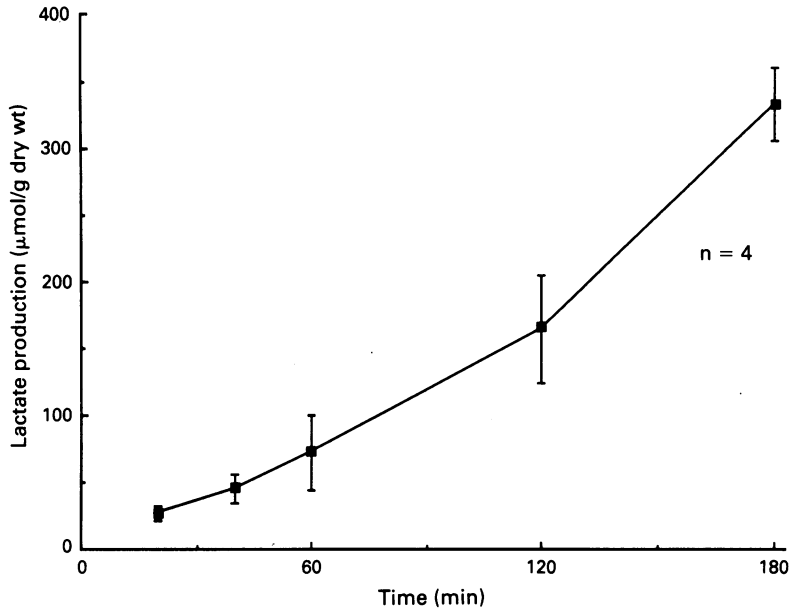

Figure 1: Lactate production by colonic epithelial cells from 5 mM glucose.

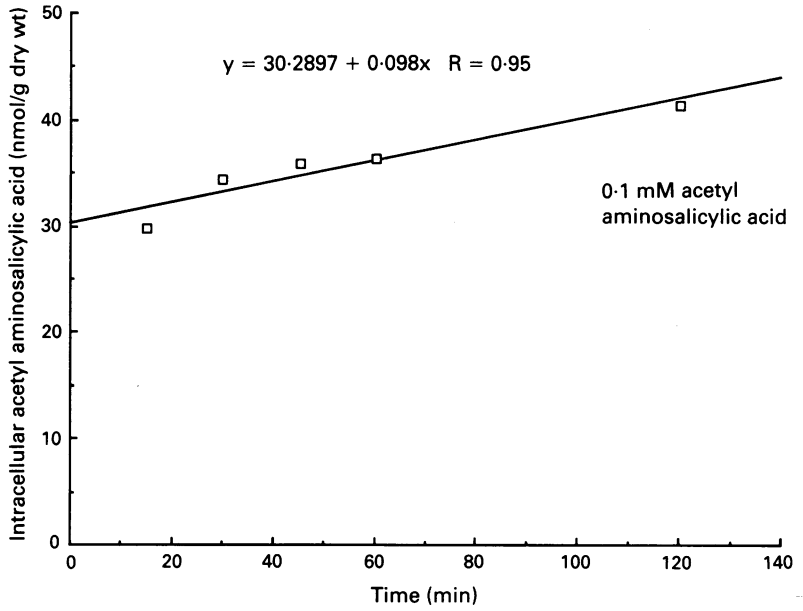

Figure 2: Time course of acetylaminosalicylic acid uptake (0·1 mM acetylaminosalicylic acid).

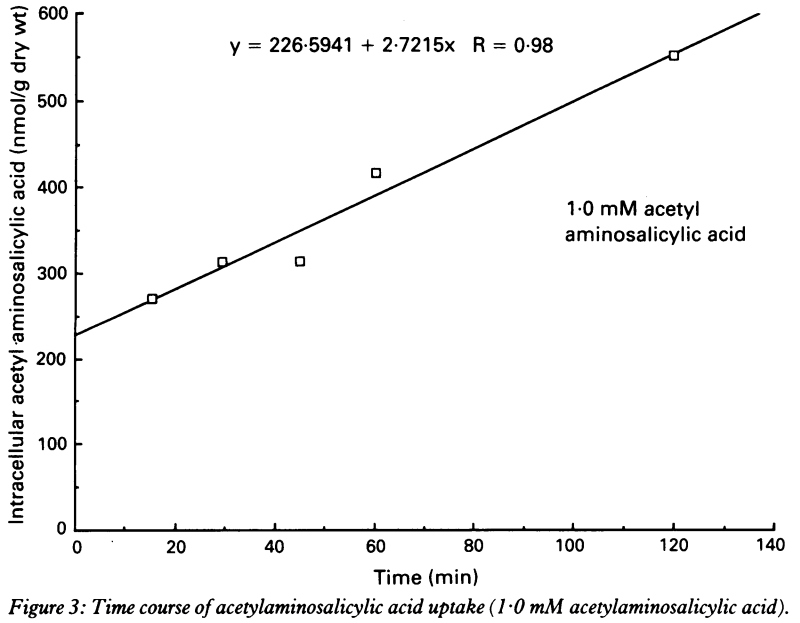

Figure 3: Time course of acetylaminosalicylic acid uptake (1·0 mM acetylaminosalicylic acid).

supernatants were filtered and analysed for the presence of Ac-ASA and 5-ASA by an HPLC method. In addition, the washings were stored and analysed for drug content by the same method.

The dry weight was calculated by passing a known volume of cell suspension through a glass fibre filter paper (Whatman) over suction (Buchner flask) and comparing this with the weight of filter paper plus the same volume of buffer after overnight drying at 120°C.

TIME COURSE OF AC-ASA UPTAKE

The time course of Ac-ASA uptake was investigated to try to differentiate between true uptake and simple entrainment of drug in the glycocalyx. Identical flasks containing cell suspension, 5 mM glucose, and 0·1 mM 5-ASA were therefore prepared and the reactions stopped by centrifugation at time intervals ranging from 0 to 120 minutes. The samples were treated as above.

WASHING EXPERIMENTS

Washing experiments were performed to try to determine whether the drug was being removed from the outside of the cell or being leeched out from the inside. Initially, the third wash was analysed for the presence of Ac-ASA. This yielded amounts of Ac-ASA up to 30% of the level subsequently found inside the cells. The number of routine washes was therefore increased. After incubation of 2 ml cell suspensions as above in identical flasks, up to six serial washes were undertaken, using 2 ml PBS, with HPLC analysis of all the washings. In addition, apparent intracellular drug concentrations were estimated after each of the washes by ultrasonic disruption of the pellet in 0·05 M phosphate buffer, as above.

Results

UPTAKE OF AC-ASA

Figure 1 shows the production of lactate from 5 mM glucose over a three hour period (mean of four experiments). Production remains linear over this time suggesting good viability, and all experiments were completed within a two hour incubation period. Trypan blue exclusion was consistently >80%. There was no relationship between lactate production and 5-ASA/Ac-ASA uptake.

The average uptake of Ac-ASA by the intact cells in one hour was 34·3 (17·5–48·3) nmol/g dry weight (n=6, mean (range)) for 0·1 mM, and 481·2 (419·2–543·1) nmol/g dry weight (n=2) for 1 mM. In those experiments in which Ac-ASA uptake was measured at both one hour and two hours (n=3, mean (range)), the results were 30·7 (17·5–38·3) nmol/g and 36·4 (20·7–47·1) nmol/g respectively, both at an incubation concentration of 0·1 mM Ac-ASA.

Figures 2 and 3 show the time course of Ac-ASA uptake over two hours for both concentrations of drug (0·1 mM and 1·0 mM) in two single experiments. As can be seen, uptake is linear after an initial high value. This may represent an

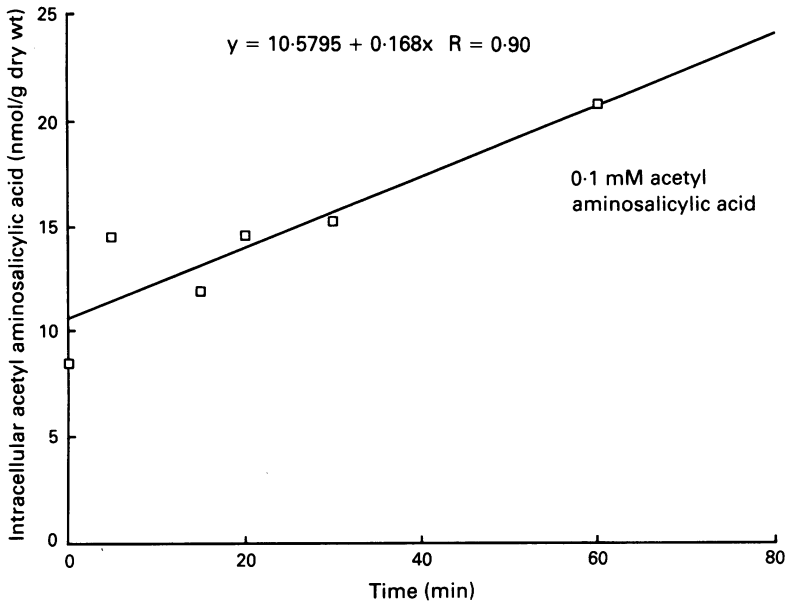

Figure 4: Time course of acetylaminosalicylic acid uptake (0·1 mM acetylaminosalicylic acid) with detection of intracellular acetylaminosalicylic acid at time 0, and earlier time points.

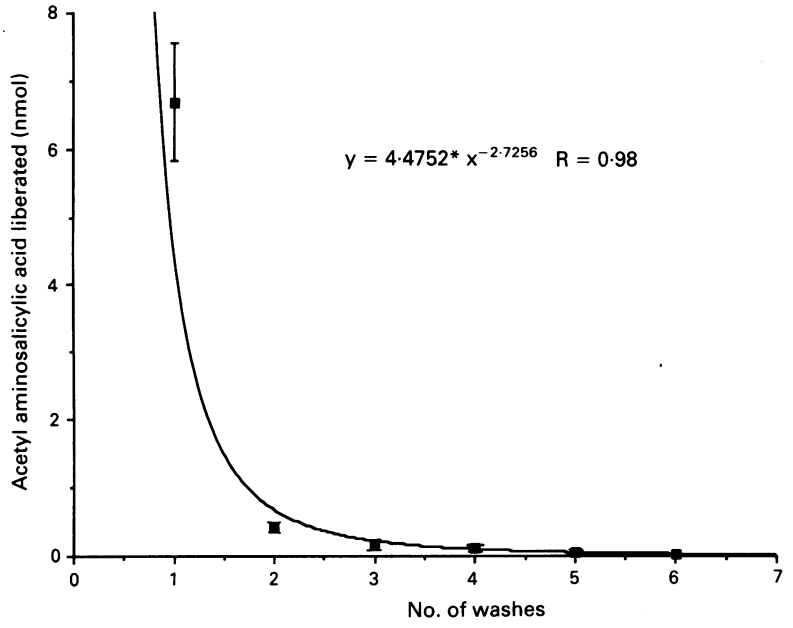

Figure 5: Acetylaminosalicylic acid content of serial washes.

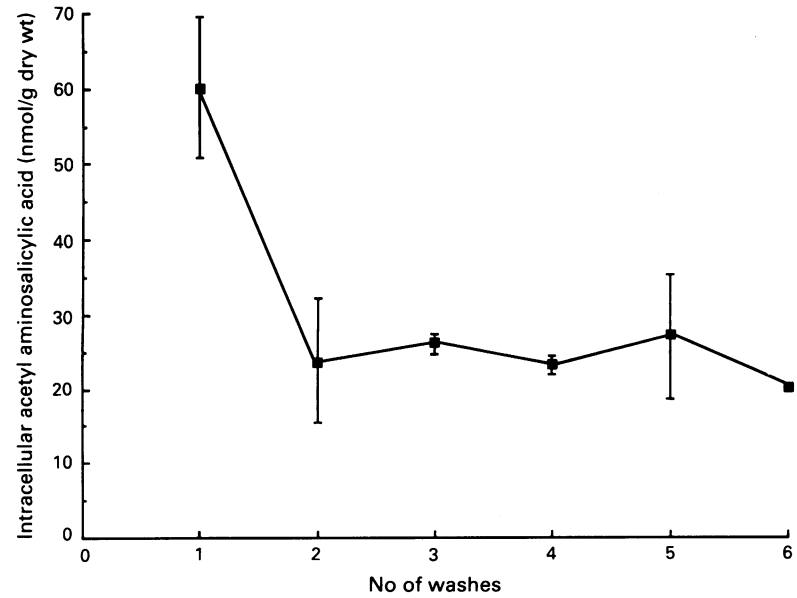

Figure 6: Intracellular acetylaminosalicylic acid content after serial washes.

initial rapid drug adherence to the cell surface followed by linear true drug uptake, and this is confirmed in Figure 4 which shows a further time-course experiment with analysis of intracellular Ac-ASA at earlier time periods, including time 0 (immediately on addition of Ac-ASA). Uptake is again linear, but even at time 0 there are significant quanities of apparent intracellular Ac-ASA. This seems unlikely to represent true uptake into the cell.

When serial washings were analysed for the presence of Ac-ASA, there was an exponential fall in concentration, compatible with removal of drug from the cell surface (Fig 5). In a pair of experiments designed to determine the amount of Ac-ASA remaining intracellularly after up to six washings, the amount of Ac-ASA present intracellularly stayed remarkably constant, following an initial drop which probably represents the removal of major surface contamination (Fig 6).

In order to compare the uptake of Ac-ASA with that of 5-ASA, the rate of uptake of Ac-ASA over one hour has to be derived. This is because the apparent uptake at one hour cannot be taken to be the true uptake in view of the high level at time 0, which must represent adherence to the cell wall (Fig 4). If the mean levels for one hour and two hours are plotted, then a line intersecting the two points can be taken back to the y-axis (Fig 7) and the hourly uptake calculated from the slope, thus:

36·4–24·9
=11·5 nmol/g over 2 hours
=5·75 nmol/g dry weight over 1 hour

UPTAKE OF 5-ASA

The uptake of 5-ASA by the intact cells has to be assumed to be equal to the amount of Ac-ASA produced and detected both extracellularly and intracellularly. This is because acetylation takes place rapidly on the entry of 5-ASA into the cell and no unchanged 5-ASA can be detected intracellularly.[4] As it has previously been shown that there is no enzyme activity in either the supernatant or the brush border,[4] the drug must be absorbed before it is acetylated. The Table shows the amount of Ac-ASA detected, both intracellularly and extracellularly, after incubation of intact cells with 0·1 mM 5-ASA (n=4).

There is a marked discrepancy between the total production of Ac-ASA, representing 5-ASA uptake (160·5 nmol/g dry weight) and uptake of Ac-ASA itself (5·75 nmol/g dry weight). Even if one assumed the uptake of Ac-ASA to be the total measured drug, including the adherent component (30·7 nmol/g dry weight), it would still be significantly inferior to the uptake of 5-ASA.

Discussion

There seems little doubt now that 5-ASA is the active moiety of sulphasalazine in ulcerative colitis.[1,12] After nearly 50 years of use, however, its mechanism of action remains obscure. In view of its poor absorption and high local concentra-

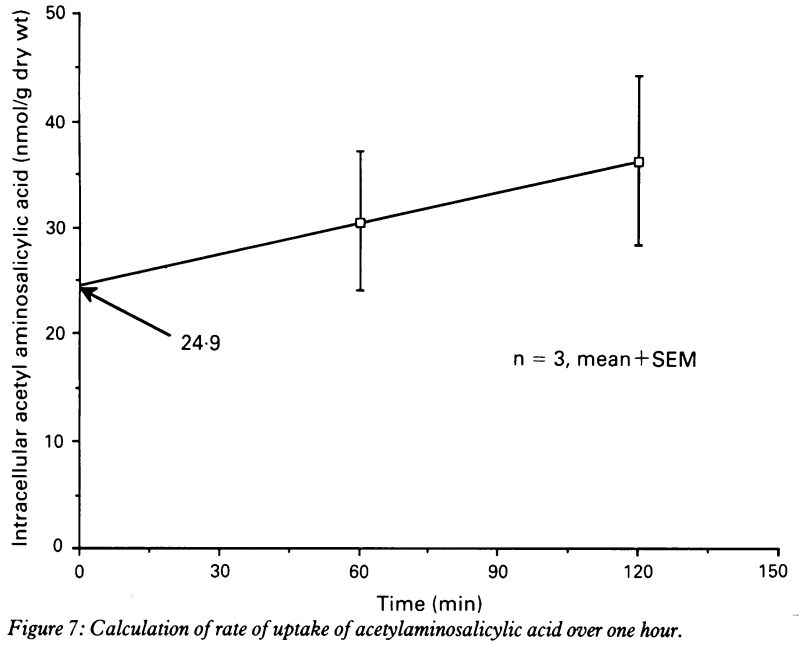

Figure 7: Calculation of rate of uptake of acetylaminosalicylic acid over one hour.

tion, its site of action seems likely to be within the bowel wall. It has been shown that 5-ASA can be detected in the colonic mucosa, but only in its acetylated form.[5] The colonic epithelial cell is probably the major site of metabolism of 5-ASA[4] and because the N-acetyltransferase enzyme is cytosolic, 5-ASA must be taken up into the cell before acetylation. It has been suggested that acetylation probably inactivates the drug, as is often the case with hepatic metabolism of drugs. However, if the drug exerts its action intramucosally after absorption, as seems likely, it is doubtful whether sufficient unchanged 5-ASA would be present within the mucosa to have any effect.[5] Ac-ASA certainly has some pharmacological effects which may be important as a proposed mechanism of action for the drug.[13,14]

Willoughby *et al*[6] found Ac-ASA to be significantly better than placebo when given rectally to patients with active proctitis. Since then, however, two further studies have shown it to be no better than placebo[7] and inferior to 5-ASA.[8] Methodological differences may partly account for the discrepancy between these studies. In contrast to the two European studies, the Oxford study[6] included 23 out of the 40 patients with sigmoidoscopic activity only. They were otherwise in clinical remission.

Another possible reason for this discrepancy may be that Ac-ASA is less well taken up by the colonic mucosa than 5-ASA, resulting in subtherapeutic mucosal concentrations. Indirect evidence for this hypothesis comes from a recent study where urinary and faecal concentrations of 5-ASA and Ac-ASA were measured after taking both 5-ASA and Ac-ASA by either the oral or rectal route in human volunteers.[9] Urinary recovery of drug was significantly greater for 5-ASA than Ac-ASA with both routes of administration and significantly more Ac-ASA was discharged in the faeces. This suggests a discrepancy in absorption in both the large and small bowel.

In the rat small intestine, many drugs are acetylated and excreted into the lumen. A dose dependent excretion of the N-acetyl forms suggests the potential saturation of N-acetyltransferase activity.[15] It has also been found in the rat that intestinal absorption rates of N-acetyl derivatives are much slower than those of the parent drug.[10] There is certainly no suggestion that 5-ASA is formed from the administered Ac-ASA, as no 5-ASA is detected in the urine or faeces after administration of Ac-ASA,[9] and in two volunteers, when deuterated Ac-ASA was administered rectally, there was no de-acetylation and re-acetylation noted.[16]

In this study, we have used the isolated human colonic epithelial cell as a means of comparing the uptake of Ac-ASA with that of 5-ASA. The colonic epithelial cell is probably the major site of acetylation of 5-ASA when the drug is delivered in an appropriate way to the colon,[4] and as the N-acetyltransferase enzyme is cytosolic, 5-ASA must be taken up by the epithelial cell before acetylation can take place. Acetylation takes place so rapidly and completely that no unchanged 5-ASA can be detected inside the cell.[4] The uptake of 5-ASA must therefore be assumed to be equivalent to the production of Ac-ASA over time. This study shows that the uptake of Ac-ASA is significantly less than that of 5-ASA. There is an initial high adherence of Ac-ASA to the cell matrix followed by a linear rise which represents true uptake by the cell. Even if one included this initial adherence, however, there would still be a significant difference in uptake, but the derived uptake is a mere fraction of that of 5-ASA.

The use of this experimental design cannot exclude the possibility that Ac-ASA is pushed back out of the cell after its uptake, as a small excretion rate could not be detected in the presence of such large extracellular quantities. It seems unlikely that significant excretion would occur against such a large concentration gradient unless the drug is actively transported out of the cell. No information is available on this.

This direct comparison confirms the results of Van Hogezand's indirect study which suggested that Ac-ASA was less well absorbed by the intestinal mucosa,[9] and shows that the colonic epithelial cell handles these compounds in a similar way to the rat small intestine.[10]

This study therefore provides a plausible reason why Ac-ASA is probably ineffective

N-acetylaminosalicylic acid (Ac-ASA) content of supernatant after incubation of intact epithalial cells with 0·1 mM 5-aminosalicylic acid (5-ASA) for one hour

Experiment no	*Ac-ASA production (nmol/g dry wt)*
1	115
2	73
3	126
4	136
Median	120·5 (range 73–136)

Intracellular Ac-ASA content after incubation with 0·1 mM 5-ASA for one hour

Experiment no	*Ac-ASA content (nmol/g dry wt)*
1	68
2	48
3	30
4	46
Median	47 (range 30–68)

Thus, total Ac-ASA production in one hour = 167·5 nmol/g dry wt

clinically when given in that form. This is unfortunate as Ac-ASA is a much more stable compound than 5-ASA and could therefore be much more easily prepared for widespread commercial use. There is, as yet, no evidence that Ac-ASA inside the mucosa is inactive, and indeed there are good theoretical reasons for assuming that the drug present in the highest local concentration is the active one. It is therefore still important to examine the role of Ac-ASA in any in vitro experiment designed to test the mechanism of action of 5-ASA and to bear in mind the differential absorption of these drugs when an intact cell system is used.

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