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THE EFFECTS OF ASPIRIN AND OTHER NON-STEROID ANTI-INFLAMMATORY/ANALGESIC DRUGS ON GASTRO-INTESTINAL MUCUS GLYCOPROTEIN BIOSYNTHESIS *IN VIVO*: RELATIONSHIP TO ULCEROGENIC ACTIONS

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Abstract—Aspirin and certain other ulcerogenic non-steroid antiinflammatory (NSAI) drugs inhibit the incorporation *in vivo* of [^{35}S]sulphate into the mucus glycoproteins isolated from the gastric mucosa of rats. Some NSAI drugs, which are shown to be relatively non-ulcerogenic in a sensitive gastric ulcer assay and the analgesic drugs paracetamol and dextropropoxyphene (which are non-ulcerogenic) did not inhibit the biosynthesis of sulphated mucus glycoproteins *in vivo*. A relationship is therefore shown between the ulcerogenicity of NSAI drugs and the inhibitory effects on gastric mucus biosynthesis. Some potent intestinal ulcerogens (e.g. indomethacin) also inhibit the incorporation of [^{35}S]sulphate into the glycoproteins from the upper intestinal mucosa. Aspirin in contrast stimulates the incorporation of [^{35}S]sulphate into intestinal mucus glycoproteins. The inhibition of the biosynthesis of sulphated gastric mucus glycoproteins by aspirin is shown to (1) depend on the presence of high concentrations of salicylates in the mucosal tissue, (2) be as a consequence of combined inhibitory capacity of aspirin, salicylate and possibly traces of metabolites in the mucosa and (3) be due to an inhibition of the sulphotransferase activity *in vitro*. Aspirin also inhibits the incorporation into gastric glycoproteins of [^{14}C]acetate, but not [^{14}C]threonine, but the effect on [^{14}C]acetate incorporation may be due to the redistribution of the isotope *in vivo*.

Inhibition of mucus glycoprotein biosynthesis has been suggested as a major factor in the development of gastro-intestinal lesions and ulceration that is induced by aspirin and related NSAI drugs [1–3]. This suggestion is based on two lines of evidence namely: (1) a reduction in the content of various carbohydrate components of mucus following repeated administration of aspirin [1–4] and (2) an inhibition by salicylates and aspirin of the *in vitro* incorporation of radioactively labelled monosaccharides (e.g. glucose *N*-acetylglucosamine), acetate or sulphate into glycoprotein fractions [5, 6]. This does not, however, provide conclusive evidence for inhibitory effects of the drug *in vivo*. Reduction in the carbohydrate content of mucus following repeated administration of the drug could be due either to an increase in the degradation rate of these glycoproteins or to other systemic actions of the drug(s). Also, the *in vitro* conditions may not give an accurate representation of the pattern of uptake and incorporation of glycoprotein components *in vivo* so making it difficult to relate any inhibitory effects to the onset of lesion development. There have been reports of salicylate inhibiting $^{35}SO_4^{2-}$ incorporation into the gastric mucosa *in vivo*, but the results are equivocal [7, 8].

In the present work, the effects of aspirin (*O*-acetylsalicylic-acid) and related NSAI drugs have been studied on the incorporation of radioactively labelled precursors into mucus glycoproteins isolated from the gastro-intestinal tract. Particular attention has been placed on the techniques for demonstrating the effects of aspirin *in vivo*, and relating the biochemical effects to metabolism; distribution of the drug and the pathogenesis of lesion development.

MATERIALS AND METHODS

In vivo *experiments*

Male Hooded rats (200 ± 10 g body wt) were starved for 24 hr and given water *ad libitum* before the experiments. The animals were given a single oral dose of aspirin or other anti-inflammatory/analgesic drugs prepared as fine suspensions in water immediately before use as previously described [9]. Following administration of the drug an i.p. injection was given of either 100 μCi of $Na_2^{35}SO_4$ (carrier-free), 20 μCi L-[U-^{14}C]threonine (180 mCi/m-mole), or 20 μCi [1-^{14}C]acetate (40 mCi/m-mole), (from the Radiochemical Centre, Amersham, U.K.) in 1 ml sterile saline. The animals were killed (at times stated in results), a blood sample collected and the stomach and upper ca. 12 cm of intestine adjacent to the stomach were removed. The gastro-intestinal tract was quickly washed free of contents with 0.9% w/v NaCl, placed into a plastic vial and immediately plunged into liquid nitrogen.

Isolation of glycoprotein fractions. Scrapings were obtained from the frozen gastric and intestinal muco-

Abbreviations—NSAI, non-steroid anti-inflammatory (drugs); EDTA, ethylene diamine tetra acetic acid disodium salt; ATP, adenosine 5'-triphosphate; PAP^{35}S, 3-phosphoadenosine-5'-phosphosulphate-^{35}S; SDS, sodium dodecylsulphate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-di(2-(5-phenyloxazolyl) benzene; TCA, trichloracetic acid; PTA, phosphotungstic acid.

sae using a microscope slide and were homogenized in 5 ml ice-cold 0.9% w/v NaCl. Subsequently a 0.1 ml aliquot of the homogenate was taken for the determination of total radioactivity. This served as a check on the total uptake of radioactivity. In some experiments a further 0.1 ml aliquot was taken for the extraction of the TCA/PTA precipitable glycoproteins [10]. Three volumes of ice-cold ethanol were added to the remainder of the homogenate and the mixture centrifuged at 2000 *g* for 10 min. The supernatant layer was discarded, 5 ml acetone was mixed with the precipitate and the mixture centrifuged (2000 *g* for 10 min). The precipitate from this was suspended in 1 ml H_2O and digested for 18 hr with 7 mg/ml papain (BDH or Calbiochem) freshly prepared in 5 ml 0.1 M citrate buffer pH 5.5 with 1 mM EDTA and 5 mM cysteine added [5]. After digestion 6 ml 1 M Na_2SO_4 was added to the ^{35}S-containing mixtures to minimize absorption of $^{35}SO_4^=$ to the dialysis or Millipore filters used subsequently for removing unwanted low mol. wt materials. For other radioactively labelled mixtures this step was omitted. The ^{35}S-labelled glycopeptides were isolated and washed free of unwanted low mol. wt substances on either Millipore filters (0.45 μm pore, Millipore Corp., MA, U.S.A.) with 30 ml aliquots of 0.1 M Na_2SO_4 + 0.1 M H_2SO_4, 0.1 M Na_2SO_4 and H_2O in succession, or dialyzed in Visking bags mounted on a shaker dialyzer against repeated changes of Na_2SO_4 then H_2O (with a few crystals of thymol added to prevent bacterial growth) for 10–14 days. The ^{14}C-labelled glycopeptides were dialyzed against repeated daily changes of H_2O with thymol added. No differences were observed in labelling or the chemical composition of the glycopeptides obtained following either of the dialysis or Millipore filtration procedures. The latter procedure was used in most of the experiments.

The glycoprotein/glycopeptide fractions isolated on the filters were dried to constant wt at 60°. Where the dialysis procedure was employed, the glycoproteins were recovered by precipitation with 3 vol. of ethanol and transferred to pre-weighed scintillation vials following suspension in a small quantity of ether. The ether was evaporated to dryness and the dry wt obtained. The glycoprotein/glycopeptide fractions were solubilized in a mixture of 0.1 ml NCS (Amersham Searle, IL, U.S.A.) + 1.0 ml H_2O and neutralized with glacial acetic acid (35 μl) after digestion. Toluene-PPO-POPOP scintillant (10 ml) was added prior to the determination of radioactive content in a Packard model 2002 Scintillation Spectrometer [12]. The radioactive counts were corrected for quenching using the channels ratio method or by internal standardization, and the ^{35}S content corrected for decay [13].

The aliquot taken for the isolation of the TCA/PTA precipitable glycoproteins was homogenized in 5 ml of a mixture of 10% w/v ice-cold trichloracetic acid and 1.0% w/v phosphotungstic acid [10]. The precipitate following centrifugation (2000 *g* for 10 min) was extracted with 5 ml quantities of ethanol and ether, suspended in a small quantity of H_2O and washed on Millipore filters (as above) prior to radioactivity determinations. The ^{14}C-labelled proteins were isolated as described [11].

Blood radioactivity. Blood samples were assayed for radioactive content following digestion in HNO_3 [14]. Aliquots (0.1 ml) of the digest were neutralized with 1.0 ml 0.75 M Tris and 0.1 ml quantities of this mixture added to 10 ml Triton-toluene PPO-POPOP scintillant for the determination of radioactivity [12].

Characterization of gastric mucus glycopeptides. Groups of 5–15 rats were given an i.p. injection of 100 μCi $Na_2^{35}SO_4$ or 10 μCi L-[U-^{14}C]threonine (as above) and killed 3 or 5 hr later. The gastric mucosal scrapings were extracted, digested with papain and then dialyzed as described above. The dialyzed fractions were freeze-dried and weighed prior to column chromatography. The fractions were chromatographed on columns (45 × 2.3 cm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) which were pre-equilibrated and eluted at room temperature with either 0.9% w/v NaCl, 0.05 M sodium borate buffer pH 8.5 (both with 0.02% w/v sodium azide added to prevent bacterial growth), or 0.1% w/v SDS and eluted at 30° to prevent aggregation of the detergent. The samples chromatographed on the SDS columns were prepared in 15 ml 1% w/v SDS with 0.2 ml 2-mercaptoethanol added and the other samples were prepared in 5 ml of the column mixture. The column fractions were assayed for radioactivity (in Triton-toluene based scintillant as above), the protein absorbance measured spectrophotometrically at 280 nm. In some experiments, the hexose and amino-nitrogen content were also determined [15, 16].

The $^{35}SO_4$ and [^{14}C]threonine labelled as well as non-radioactive fractions from papain digestion were subjected to electrophoresis in the agarose-polyacrylamide gel system of Holden *et al.* for 5–15 min at 12.5 mA per tube and a potential difference of 110 V [17]. The gels were stained with periodic acid-Schiff's reagent, Alcian Blue, periodic acid followed by Alcian Blue, Napthalene Blue-Black, or Toluidine Blue [18–22]. A blank was included (with no sample but agarose added to the sample layer) with each run. Following electrophoresis of the radioactively-labelled glycopeptides the gels and sections of the blank gels were sliced into 1.0 mm sections and the radioactive content in each slice determined after solubilizing in 0.1 ml NCS and mixing with 10 ml toluene PPO-POPOP scintillant [12].

The freeze-dried fractions from papain digestion were also assayed for amino-nitrogen and hexose content [16, 17].

Gastric ulcer assays. The gastric damage induced by the NSAI drugs was assayed using a stress-sensitizing procedure described previously [23]. In this method the rats are exposed to brief periods of cold stress (−15°, 45 min) following oral administration of the drugs. This assay markedly increases the sensitivity of the gastric mucosa to the NSAI drugs with known ulcerogenic activity but is not sufficient to induce the development of gastric damage in control animals exposed to cold stress *alone* [23]. The drugs were prepared as fine suspensions in water by homogenizing immediately before use. Aliquots (1 ml) of the drug suspension were given orally by a 1 ml tuberculin syringe with a cannula attached to a sixteen gauge needle [9]. The rats were killed 2 hr after drug administration and the number of lesions and their severity (assessed on an arbitrary scale of 0–4+) of mucosal damage was determined by visual examin-

ation with the aid of a 4× illuminated magnifying lens. The ulcer index was calculated by summing the number of lesions [9], their severity and the number of animals with mucosal damage in each treatment group × 10 (the mean number of lesions is directly related to the ulcer index). The effects of the NSAI drugs on mucus biosynthesis were then compared with the gastric ulcer indices.

The distribution of salicylates in the gastrointestinal tract and mucosae following oral dosing of aspirin

The concentrations of salicylates in the blood, gastro-intestinal mucosae and tract were measured following the oral administration of 200 mg/kg [^{14}C]carboxyl labelled-aspirin (The Radiochemical Centre, Amersham, U.K.) as 1 ml suspension in H_2O. The animals were killed at time intervals ranging from 10 min to 2 hr after dosing and samples of blood collected. The stomach and upper 12 cm of intestine proximal to the stomach were excised separately and the contents of the stomach and the intestinal tract were washed out with 0.9% w/v aqueous NaCl and made up to a final volume of 10 ml. The blood, gastric and intestinal contents and tissues were placed in plastic vials and immediately plunged into liquid nitrogen. The samples were stored at −15° after collection or between preparation and extraction procedures to minimize the hydrolysis of aspirin.

Homogenates of the gastric and intestinal mucosae were prepared from scrapings of the frozen tissue and after homogenizing the scrapings in 2 ml of ice-cold 0.9% w/v aqueous NaCl, a 0.10 ml aliquot of the homogenate was obtained for the determination of total [^{14}C]salicylates [13]. Immediately afterwards 3 vol. of ice-cold ethanol was added and the mixture was centrifuged (2000 *g* for 10 min). The supernatant was stored on ice and the pellet resuspended in 5 ml ice-cold 80% v/v ethanol and centrifuged (2000 *g* for 10 min). The combined supernatants were evaporated down *in vacuo* and resuspended in 1.0 ml of 80% v/v ethanol. A 0.1 ml aliquot of this extract was placed in 10 ml Triton X-100 toluene PPO-POPOP scintillant with 1.0 ml H_2O added for determining the total radioactivity in tissues [12].

The concentrations of acetylsalicylic and salicylic acids in aliquots of the ethanolic extracts or gastrointestinal contents were determined, by thin layer chromatography on Merck Silica gel GF_{254} aluminium sheets in petroleum ether (40–60°b.p. range)–propionic acid (10:1) [24]. The radioactive spots on the t.l.c. plates were detected using a Birchover t.l.c. scanner or by co-chromatography with authentic standards. The quantity of radioactivity present was obtained by scraping off the spots, suspending the scrapings in 1.0 ml H_2O and counting in 10 ml of Triton-toluene PPO-POPOP scintillant [12].

The total content of [^{14}C]salicylates in the blood and mucosal homogenates were determined as for the glycoprotein biosynthesis experiments [14]. Aliquots (1.0 ml) of the gastrointestinal contents were assayed for radioactivity in 10 ml Triton toluene PPO-POPOP scintillant [12]. The concentrations of [^{14}C]salicylates were determined after correction for quenching [13] by comparing the radioactive content in the tissues with that in an aliquot of the original radioactive aspirin as prepared for dosing.

In vitro *experiments*

Scrapings from rat gastric mucosa were incubated in a medium comprising: 2 ml Kreb's medium III [25], 0.1 ml drug solution (as sodium salts) or an equivalent concentration of NaCl (as a control), and 0.1 ml of either 10 μCi $Na_2^{35}SO_4$ (carrier-free), 1 μCi [1-^{14}C]acetate (sodium salt, 40–60 mCi/m-mole), 1 μCi 1-[U-^{14}C]threonine (180 mCi/m-mole) or 1 μCi D-[1-^{14}C]galactosamine −HCl (45 mCi/m-mole). The incubations were started by the addition of 0.2 ml scrapings (delivered from a large bore 2 ml plastic syringe). After incubation for 1 hr at 37°, the incubations were terminated by the addition of 3 vol. of ethanol and allowed to stand overnight at −15°. Following thawing, the mixture was centrifuged (2000 *g* for 10 min), the precipitate homogenized in 5 ml ice-cold 0.9% w/v NaCl and the radioactivity determined in the isolated glycoproteins (as above).

Effects of aspirin and salicylate on sulphate transfer from PAP^{35}S into glycoproteins in vitro. The gastric mucosa from 30 male rats was scraped and homogenized in 10 vol of ice-cold medium comprising 0.02 M Tris–HCl buffer pH 7.4 with 0.113 M KCl and 3 mM $MgCl_2$ added [26]. The homogenate was centrifuged at 105,000 *g* for 60 min (4°) in a Spinco model L ultracentrifuge and the supernatant withdrawn for incubation. The incubation medium comprised 2 ml 105,000 *g* supernatant, 0.1 ml of 2 μCi PAP^{35}S (New England Nuclear, Boston, specific activity 5.96 Ci/m-mole, Lot 897–144), 0.15 ml drug (freshly prepared as sodium salts) to give final concentration of 0.5–10 mM. Corresponding concentrations of NaCl were added to control tubes. The incubations were performed for 1 hr at 37° and were terminated by the addition of 3 vol. of ice-cold ethanol. After extraction with acetone the glycoprotein fractions were separated using the Millipore filtration procedure and radioactivity determined as described above.

RESULTS

Characterization of the products from papain digestion. The results from the chromatography of the $^{35}SO_4^=$ and [^{14}C]threonine labelled glycoprotein mixtures are shown in Figs 1a, b. Preliminary attempts to chromatograph the papain digest mixtures of gastric mucosae on Sepharose 4B columns eluted with 0.9% NaCl were unsatisfactory since a considerable quantity of material was retained at the top of the column even with dilute mixtures. Successful chromatography was achieved by employing dilute mixtures eluted with 0.05 M sodium borate, pH 8.5. However, more satisfactory separation was achieved on columns eluted with 0.1% SDS and larger quantities of the glycoprotein mixtures could be applied to these columns (Figs 1a, b). The results show that the major peak of $^{35}SO_4^=$ labelled material (component A, Fig. 1a) was separated from that of one of the major protein absorbing peaks on the columns eluted with 0.1% SDS whereas these two peaks appeared to coincide on the columns eluted with 0.05 M sodium borate.

The major component (A of Figs 1a, b) of $^{35}SO_4^=$ labelled material observed in chromatograms from columns eluted with SDS appeared to have a low peptide content but an appreciable quantity of hexose

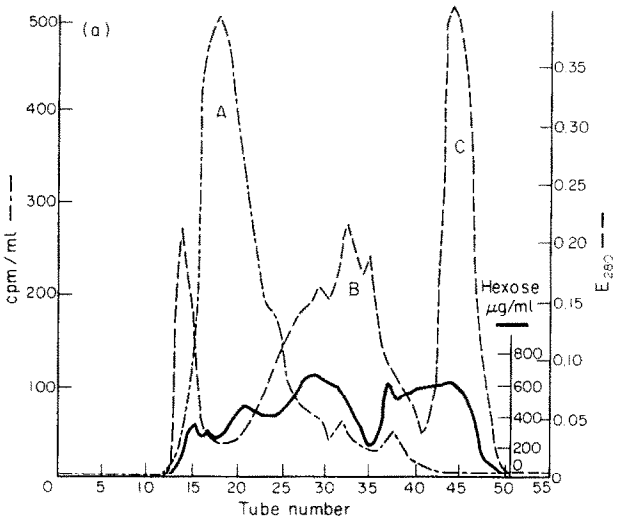


Fig. 1a. Column chromatography of ^{35}S-labelled glycoprotein fractions. A sample containing 46,150 dpm ^{35}S from papain digestion and dialysis was applied to a column of 45 × 2.3 cm of Sepharose 4B and eluted with 0.1% SDS and 10 ml (approx.) fractions collected.

(Fig. 1a). When these profiles (Fig. 1a) are compared with the chromatograms of [^{14}C]threonine labelled materials (Fig. 1b) it appears that the major $^{35}SO_4^=$ labelled peak corresponds with two which are labelled with [^{14}C]threonine. These results show that papain digestion and dialysis of the gastric mucosa of rats yields, amongst other products a major component which are sulphoglycopeptides according to established criteria [27]. Also, it appears that there is a small quantity of $^{35}SO_4^=$ labelled glycopeptides which trail away from the main labelled peak (A) suggesting, that there is present a heterogeneous collection of glycopeptides of varying extent of sulphation. This is also supported by the results from the electrophoresis of the labelled papain digests under dissociating conditions (i.e. with SDS + mercaptoethanol present). Here two major $^{35}SO_4^=$ and [^{14}C]threonine labelled components with mobilities of 2.2 cm and 3.6 cm (total tube length, 6 cm) were observed after electrophoresis for 15 min and the latter stained heavily with both Alcian Blue and Napthalene Blue-Black. Other minor components usually 2 or 3, were also evident but only in trace quantities and these also had trace labelling.

Effects of aspirin and salicylic acid on glycoprotein biosynthesis in vivo. The results (Table 1) show that oral administration of aspirin at a dose of 200 mg/kg caused a marked inhibition of the incorporation of $^{35}SO_4^=$ into isolated gastric glycoproteins at time intervals of 5 hr or later following administration of the drug. In contrast, inhibition of $^{35}SO_4^=$ incorporation was only evident 24 hr after administration of the same dose of salicylic acid. It is also apparent that maximal inhibition occurred at the time of maximal $^{35}SO_4^=$-incorporation i.e. between 5–24 hr (Table 1). In preliminary studies it was established that there

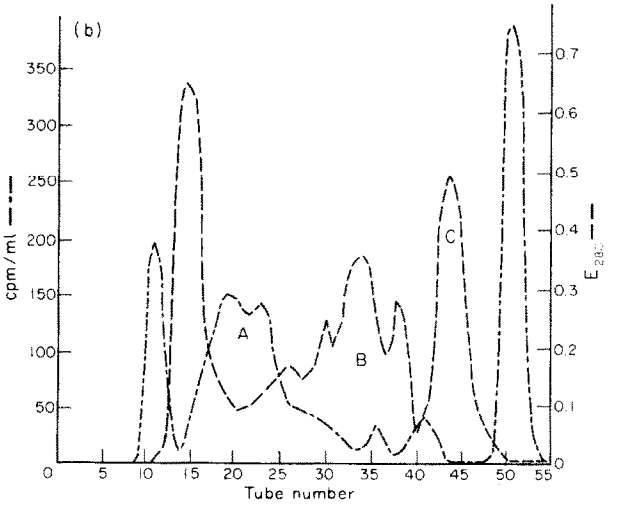


Fig. 1b. Column chromatography of [U-^{14}C]threonine labelled glycoproteins. A sample containing 16,650 d.p.m. radioactive labelled material from papain digestion and dialysis was chromatographed in the same column as in Fig. 1a.

Table 1. Effects of aspirin or salicylic acid on the incorporation of $^{35}SO_4^{2-}$ into isolated rat gastric mucosal glycoproteins *in vivo* with time

Time of Drug Treatment†	Drug	Dose mg/kg	dpm/mg dry wt Control (mean ± S.D.)	dpm/mg dry wt Drug-treated (mean ± S.D.)	't'	% change
1 hr	Aspirin	200	703 ± 169(5)‡	773 ± 80	0.49	0
2 hr		200	1283 ± 315(4)‡	1470 ± 374(5)	0.77	0
3 hr		100	4319 ± 3928(8)‡	6617 ± 2583(11)	1.54	0
5 hr		200	9363 ± 1416(6)‡	3197 ± 3036(6)	4.51*	−66
5 hr§		200	3723 ± 1866(6)	1506 ± 557(5)	2.55*	−60
1 hr	Salicylic	200	9604 ± 2400(6)	9357 ± 1161(5)	0.21	0
3 hr	acid	200	13374 ± 5167(6)	11497 ± 4426(6)	0.68	0
5 hr		200	11377 ± 2245(5)	14218 ± 2361(6)	1.83	0
24 hr		200	8174 ± 2474(5)	3588 ± 449(5)	4.08*	−56

Rats were dosed orally [9] with 200 mg/kg body wt of drug. Immediately afterwards an i.p. injection of 100 μCi Na_2 $^{35}SO_4$ was given except (§) which received isotope 5 min after aspirin administration. The animals were killed at stated times (†) and the radioactivity in the isolated mucus glycoproteins determined following papain digestion. Values in parenthesis represent the number of animals per group. * Statistically significant difference (Student's 't' test, $P \leq 0.05$) between control and drug-treated animals.

‡ Data from same experimental group, i.e. same batch of animals and isotope to show time course of $^{35}SO_4^{2-}$-incorporation in both control and aspirin-treated groups.

was a broad maxima of $^{35}SO_4^{=}$ incorporation over this time interval which fell rapidly after approximately 30 hr.

Varying the time of $^{35}SO_4^{=}$ injection, 1–5 min following the oral dose of aspirin (200 mg/kg) resulted in the same degree of inhibition (Table 1). No statistically significant effects were observed when longer time intervals were employed between the aspirin dose and the radiosulphate injection in control animals, i.e. at 0.5–7 hr (data not shown). However, a transient increase in $^{35}SO_4^{=}$ incorporation into intestinal glycoprotein was observed at the 2.5 hr time interval separating the dose of aspirin and $^{35}SO_4^{=}$ injection. The mean ± S.D. of the values for incorporation of $^{35}SO_4^{=}$ into papain fractions being: control = 695 ± 305 dpm/mg dry wt ($N = 5$), 200 mg/kg aspirin = 1455 ± 589 dpm/mg dry wt ($N = 5$), giving a statistically significant (Student's $t = 2.56$, $P \leq 0.05$) increase of 209%. No other statistically significant changes were observed in $^{35}SO_4^{=}$ incorporation in the intestinal mucosa of animals given aspirin or salicylic acid. A similar reduction of $^{35}SO_4^{=}$ incorporation occurred in the TCA/PTA extracted glycoproteins (results not shown).

In contrast to the above results the oral administration of 200 mg/kg aspirin failed to inhibit the incorporation of L-[U-^{14}C]threonine or [1-^{14}C]acetate into proteins or glycoproteins respectively when these labelled precursors were given i.p. 5 min after the drug (Table 2). To consider the possibility that the diverse systemic effects of salicylates may alter the pattern or organ uptake or metabolism of labelled acetate, experiments were performed in which the radioactive acetate was given *per os* 5 min after the dose of aspirin (or H_2O in controls). The results in Table 2 show that aspirin inhibited the incorporation of radioactive acetate when the label was given p.o. However, the total uptake of [1-^{14}C]acetate by the gastric mucosa tissue was also reduced under these conditions (Table 2) suggesting that the apparent inhibitory effect could be due to variable effects of the drug on uptake of the radioactive acetate.

Effects of NSAI/analgesic drugs on $^{35}SO_4^{=}$ incorporation into gastrointestinal glycoproteins in vivo. The

Table 2. Effects of aspirin administration on the incorporation of [1-^{14}C]acetate or L-[U-^{14}C]threonine into isolated rat gastric mucosal glycoproteins/proteins *in vivo*

Labelled precursor and route of administration	Time†	dpm/mg (mean ± S.D.) Control	Aspirin	't'	% change
[1-^{14}C]Acetate i.p.	1 hr	373 ± 248	441 ± 535	0.20	0
[1-^{14}C]Acetate p.o.‡	1 hr	13928 ± 8457	2797 ± 748	2.98	−80%
[1-^{14}C]Acetate i.p.	2 hr	243 ± 62	210 ± 50	0.90	0
L-[U-^{14}C]Threonine i.p.	1 hr	2483 ± 205	2258 ± 579	0.26	0
L-[U-^{14}C]Threonine i.p.	4 hr	3805 ± 828	4230 ± 301	1.13	0

Rats were given a single oral dose of 200 mg/kg aspirin p.o. [9] followed 5 min later by a dose of 40 μCi [1-^{14}C]acetate i.p. or p.o., or an i.p. injection of 10 μCi [U-^{14}C]threonine. The animals were killed at stated times (†) and the radioactivity in the [1-^{14}C]acetate labelled glycoproteins determined following papain digestion and that in [U-^{14}C]threonine labelled proteins determined after TCA precipitation and extraction [11]. The total uptake of radioactive precursors into homogenates was unchanged in all except the group (‡) given [1-^{14}C]acetate p.o. where a statistically significant reduction of 77 per cent occurred in isotope incorporation; the values being for the control (mean ± S.D.) = 1813 ± 640 ($N = 5$) and aspirin-treated group = 427 ± 129 ($N = 5$), (Student's 't' test = 4.8; $P < 0.05$).

Table 3. Effects of analgesic and/or anti-inflammatory drugs on the incorporation of $^{35}SO_4^{2-}$ into isolated rat gastric mucosal glycoproteins *in vivo*

Ulcerogenic status	Drug	Dose mg/kg	^{35}S d.p.m./mg (mean ± S.D.) Control	Drug	't'	% change	Ulcer index
1. High	Aspirin†	200	—	—	—	—	61.0
	Diclofenac	10	11109 ± 2006	8017 ± 695	2.94*	−28	44.2
	Flurbiprofen	100	929 ± 445	249 ± 122	3.30*	−27	36.0
	Indomethacin	10	11109 ± 2006	5433 ± 1805	3.94*	−51	48.0
	Suprofen	100	5213 ± 377‡	4119 ± 372	4.36*	−21	61.0
2. Low/ moderate	Azopropazone	100	6579 ± 3073	5974 ± 1420	0.39	0	0
	Dextropropoxyphene napsylate	200	1200 ± 683	1498 ± 680‡	0.60	0	0
	Diflunisal	100	2485 ± 1335	6833 ± 5247‡	1.81	0	15.1
	Fenclofenac	200	11109 ± 2006	7403 ± 3033	1.82	0	0
	Flufenamic acid	100	1200 ± 683	1414 ± 1206	0.32	0	16.8
	Naproxen	100	2485 ± 1335	3154 ± 1020	0.89	0	18.6
	Paracetamol	200	1200 ± 683	2227 ± 1800	1.08	0	0
	Salicylic acid†	200	—	—	—	—	18.8
	Sulindac	50	2485 ± 1335	2894 ± 1137	0.52	0	8.8

Drugs were given p.o. [9] to rats (at doses approximating published values of anti-inflammatory activity in the carrageenan assay) followed by an i.p. injection of 100 μCi (approx.) $Na_2^{35}SO_4^{2-}$. The animals were killed 20 hr later and the radioactivity determined in the mucus glycoprotein fractions following papain digestion. Data is from five animals per group except (‡) where four animals per group were used.

* Statistically significant reduction in $^{35}SO_4^{2-}$ incorporation (Student's 't' test, $P \leq 0.05$) compared with control value.

† Data on $^{35}SO_4^{2-}$ incorporation in Table 1.

results in Table 3 show that of the non-steroid anti-inflammatory drugs which were tested (approx. the highest therapeutic doses) that indomethacin, diclofenac, flurbiprofen, and suprofen caused in inhibition of $^{35}SO_4^{=}$ incorporation into gastric mucosal glycoproteins; the remainder having no statistically significant effects. When correlated with the gastric ulcer indices of these compounds (Table 3), it is evident that the more potent ulcerogenic drugs caused a statistically significant reduction in radiosulphate

Table 4. Effects of salicylates on the incorporation of [1-^{14}C]acetate, [U-^{14}C]threonine, $^{35}SO_4^{2-}$, and PAP^{35}S into isolated gastric mucosal glycoproteins *in vitro*

Expt. No.	Labelled precursor	Treatment	d.p.m./mg mean ± S.D.	't'	% change
1.	[U-^{14}C]Threonine	Control	70.0 ± 25.3		
		10 mM Aspirin	48.0 ± 7.5	1.87	0
		10 mM Salicylate	37.5 ± 8.2	2.74*	−46
2.	[1-^{14}C]Acetate	Control	63.0 ± 21.1		
		10 mM Aspirin	23.9 ± 8.9	3.82*	−62
		10 mM Salicylate	18.5 ± 2.9	4.69*	−71
3.	$^{35}SO_4^{2-}$	Control	130.5 ± 35.0		
		10 mM Aspirin	57.3 ± 22.0	3.15*	−56
		10 mM Salicylate	41.5 ± 21.0	4.36*	−68
		5 mM Salicylate	45.6 ± 55.5	2.59*	−65
		2 mM Salicylate	88.3 ± 38.8	1.69	0
		10 mM Salicylurate	20.5 ± 12.7	6.60*	−84
		10 mM Gentisate	15.0 ± 1.7	14.6*	−88
4.	PAP^{35}S	Control	1747 ± 11.2		
		10 mM Aspirin	942 ± 94.6	12.3*	−46
		10 mM Salicylate	284 ± 12.7	19.3*	−83
		Control	1313 ± 13.9		
		0.5 mM Salicylate	710 ± 41.0	27.9*	−46

In experiments numbered 1–3, scrapings from rat gastric mucosa were incubated in a mixture comprising: 2 ml Kreb's medium 3 [25], 0.1 ml concentrated drug solution, 0.2 mg mucosal scrapings and 0.1 ml isotopically labelled precursor. The incubations were performed at 37° for 1 hr and terminated by the addition of 3 vol. of ethanol. The radioactivity in the glycoprotein fraction was determined after extraction and papain digestion. In experiment 4, 2 ml aliquots of the 105,000 *g* supernatant fraction of rat gastric mucosa prepared in Tris KCl $MgCl_2$ medium [26] were incubated for 1 hr at 37° in a mixture with 0.1 ml of 2 μCi PAP^{35}S (0.3 μmole) and 0.15 ml concentrated drug mixture added. The reaction was terminated by the addition of 3 vol. of ethanol and the radioactive content determined in the isolated glycoprotein fraction.

* Statistically significant reduction (Student's 't' test, $P \leq 0.05$, 5 incubations per treatment group) in the incorporation of radioactive precursor in drug-treated compared with control groups. All drugs prepared as sodium salts immediately before incubation.

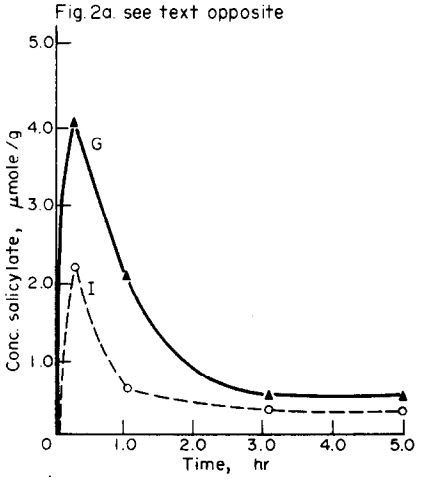
incorporation whereas those with little or no ulcerogenic activity did not. It appears therefore that there is some relationship between the degree of ulceration and inhibitory effects on gastric glycoprotein biosynthesis. Also, of the drugs tested, diclofenac and the potent intestinal ulcerogen, indomethacin, both caused a statistically significant reduction in incorporation of $^{35}SO_4^=$ into isolated intestinal glycoproteins. The mean ± S.D. values obtained being: Control = 11839 ± 1700, indomethacin (10 mg/kg) = 5678 ± 2089 (Student's 't' = 4.15, $P \leqslant 0.05$, $N = 5$), diclofenac (10 mg/kg) = 7566 ± 326 ($t = 4.28$, $P \leqslant 0.05$, $N = 5$).

The effects of salicylates on glycoprotein biosynthesis in vitro. The results (Table 4) show that all the salicylates tested caused an inhibition of $^{35}SO_4^=$ incorporation into glycopeptides isolated from the gastric mucosa incubated *in vitro*, with salicylate being a more potent inhibitor than aspirin. Notable differences in the effects of these drugs were observed in the incorporation of L-[U-^{14}C]threonine into gastric proteins since of these only salicylate (at 10 mM concentration) caused any statistically significant inhibition (Table 4). By contrast, about the same inhibition of [1-^{14}C]acetate incorporation into gastric glycoproteins was obtained with aspirin and salicylate (Table 4).

Salicylate or aspirin at 0.5–10 mM inhibited the incorporation of $^{35}SO_4^=$ from PAP ^{35}S into the isolated glycoprotein macromolecular fraction (Table 4) showing that the inhibitory effects of these drugs on the incorporation of $^{35}SO_4^=$ *in vivo* could be due to an inhibition of the enzyme (sulphotransferase) involved in the transfer of sulphate from activated precursor.

Uptake and metabolism of radioactive aspirin. The results show that maximal gastric mucosal tissue concentrations of salicylates were achieved at 10–15 min after oral administration of 200 mg/kg of the drug (Fig. 2). This coincided with a peak of salicylates in the blood (Fig. 2). It is evident that much lower concentrations of salicylates are present in the intestinal contents and mucosa compared to that in the stomach (Fig. 2). The results also show that there is rapid and appreciable rate of hydrolysis of aspirin to salicylate in the gastric mucosa (Fig. 2). When related to the results (Table 1) of the effects of aspirin on radiosulphate incorporation *in vivo* it is seen that inhibition of incorporation only occurred at time intervals (0–5 min) of maximal absorption of the drug into the gastric mucosa. Lower levels of the drug in the intestine were apparently insufficient to cause inhibition of $^{35}SO_2^=$ incorporation *in vivo* (Fig. 2). The results show that it is the cumulative absorption of aspirin over the 5 hr period which accounts for the inhibitory effects on $^{35}SO_4^=$ incorporation *in vivo*. At time intervals >5 min after administration of aspirin

Fig. 2a. see text opposite



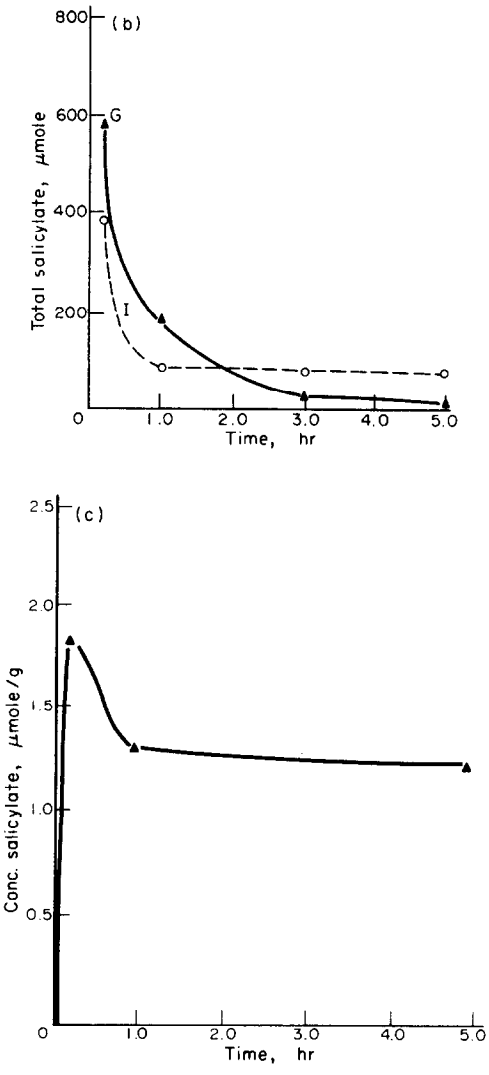


Fig. 2. Concentration of salicylates in the gastric [(G) solid line] and intestinal [(I) dotted line], mucosae as μmole/g wet wt of tissue (Fig. 2a) and luminal contents as total μmoles present (Fig. 2b) up to 5 hr following oral administration of 200 mg/kg [^{14}C]carboxy-labelled aspirin. Concentrations of salicylates in the blood are shown in Fig. 2c. Highest concentrations of salicylates are present in the gastric mucosa in the period up to 10 min following oral administration of aspirin which coincides with the time in which inhibition occurs in the $^{35}SO_4^{2-}$ incorporation into isolated gastric but not intestinal glycoproteins *in vivo* (Tables 1 and 2). Rapid metabolism of aspirin to salicylate occurs within the first 10 min as only 5–8 per cent aspirin is present in the mucosa in the period of 10 min to 5 hr compared with >90 per cent aspirin in the lumen (results not shown).

there is apparently insufficient drug absorbed in the gastric mucosa to cause inhibition of $^{35}SO_4^=$ incorporation.

DISCUSSION

In previous studies the effects of salicylate administration of $^{35}SO_4^=$ incorporation into rat gastric tissues *in vivo* produced results which can only be regarded as equivocal [7, 8]. These differences could be due to technique, especially since in one study the techniques of isolation and characterization of the radioactively labelled components were not mentioned [7]. It is important to recognize that the reduction in radiosulphate incorporation may lead to significant changes in the total amount of sulphated mucopolysaccharides/glycopeptides which can be isolated using cationic detergents as employed in another study [8]. Also, a reduction may occur in the number of residues (e.g. amino sugars) available for accepting sulphate groups on glycoproteins in the presence of the drug, so that it is necessary to isolate all the glycoproteins present which are likely to serve as acceptors for sulphate or acetate [5, 28]. In the present study a whole spectrum of glycoprotein components was found in the papain digest fractions and moreover these showed a variable degree of sulphation (Figs 1a, b). Sensitive detection of changes in the radiosulphate content should thus be possible under the conditions as employed.

The main conclusion which arises from the present work is that NSAI drugs which are particularly ulcerogenic (e.g. aspirin, indomethacin and diclofenac) cause a marked inhibition on the $^{35}SO_4^=$ incorporation into isolated glycoproteins *in vivo* whereas non-ulcerogenic NSAI drugs (e.g. fenclofenac) and analgesics (e.g. paracetamol and dextropropoxyphene) do not (Tables 1 and 3). The ulcerogenic group of drugs also depressed the incorporation of $^{35}SO_4^=$ into intestinal glycoproteins *in vivo*, with the exception of aspirin which had a stimulatory effect (see Results). The differences in the effects of these drugs on radiosulphate incorporation may be related to their ulcerogenic capacity in the intestine; indomethacin is a very potent intestinal ulcerogen whereas aspirin is not.

The results of the studies of the time course of effects of aspirin (Table 1) when related to the tissue distribution of aspirin (Fig. 2) show that aspirin only inhibits radiosulphate incorporation when this label was given at times when (i.e. over the 5 hr period of $^{35}SO_4^=$ incorporation) high concentrations of the drug have accumulated in the gastric mucosal tissue (Fig. 2 and Results). The low concentrations of the drug found in the intestinal mucosa are evidently insufficient to cause any effects on mucus biosynthesis (i.e. which is not obvious enough to be detected *in vivo*).

It appears from the results (Fig. 2) of the aspirin distribution studies that aspirin is quite rapidly metabolized to salicylate following absorption by the gastric mucosa. Any inhibitory effects of the drug on glycoprotein biosynthesis *in vivo* may be a reflection of the combined effects of aspirin, salicylate and perhaps other trace quantities of metabolites [24]. In Table 4, it is apparent that marked differences are evident in the inhibitory effects of the various salicylates on the incorporation of radioactively labelled sulphate, threonine and acetate *in vitro*, with salicylate having more marked inhibitory effects than aspirin. The absence of any inhibitory effect of aspirin on [^{14}C]threonine incorporation into gastric glycoproteins (which is observed *in vitro* concentrations of the drug which are high by comparison *in vivo*, Fig. 2) is also shown to be true *in vivo* (Tables 2 and 4), even though some salicylate would be expected to be present in the gastric mucosa (Fig. 2). The incorporation of radioactive acetate is apparently inhibited by aspirin *in vivo* when the isotope is administered in such a way as to minimize other systemic effects of the drug on the metabolism of acetate. Clearly, both aspirin and salicylate have the potential to inhibit acetate incorporation into gastric glycoproteins as seen from the *in vitro* studies (Table 4).

Thus, it appears that the most potent effects of aspirin are probably the biosynthesis of sulphated glycoproteins. The results in Table 4 show that both salicylate and aspirin markedly inhibit the transfer of sulphate from "activate sulphate" (i.e. PAP^{35}S) into the glycoprotein fractions, thus suggesting that these drugs inhibit the sulphotransferase enzyme. It is also possible that these drugs may act to inhibit mucus glycoprotein biosynthesis *in vivo* by decreasing the amount of ATP produced because of the effects of salicylate on uncoupling of mitochondrial (oxidative) phosphorylation [29]. However, this is probably only a minor effect of aspirin since this drug does not itself cause uncoupling *in vitro* and the salicylate produced after hydrolysis of aspirin in the gastric mucosa may cause only limited effect on ATP production [30].

As a possible explanation for the differences in the time course of effects of salicylate inhibition on radiosulphate incorporation into gastric glycoproteins compared to that of aspirin, it is possible that these could have a mechanistic basis in the combined and relative effects of these drugs on ATP production, PAPS formation and sulphotransferase activity. It may be that aspirin has more pronounced affects (i.e. at earlier times) on radiosulphate incorporation by affecting PAPS formation (i.e. sulphokinase reactions) despite there being lesser effects on ATP production *in vivo*. Salicylate, in contrast, may have lesser effects on sulphokinase activity despite effects on ATP production (by uncoupling) and sulphotransferase activity. The more pronounced reduction in the amount of "active sulphate" (PAPS) generated in mucosal cells by aspirin cf. salicylate would delay the inhibitory effects of the drug on sulphate incorporation into gastric glycoproteins.

The consequences of an inhibition in the biosynthesis of sulphated glycoproteins by aspirin are seen in a marked reduction in the total content of sulphated mucus components [4]. The reduction in these components and in acetylated sugar components (i.e. from inhibition of [^{14}C]acetate incorporation) may promote the development of gastric damage in ulceration; the association between gastric ulcerogenicity and capacity of an NSAI drug to inhibit mucus glycoprotein biosynthesis is seen clearly in the present work. This may have three consequences that must be considered in seeking the mechanism of gastric damage by these drugs. First, the reduction in the total number of acidic (e.g. sulphate, and sialic acid)

groups in mucus may decrease the capacity of mucus to act as a buffer for hydrogen ions. This would be expected to be important at the mucus-lined interface between the surface cells lining the stomach and the lumen where the layer is constantly being replenished following removal by abrasive food particles etc. This mucus interface would be expected to act (possibly in a dynamic sense) in forming a buffer layer so generating a pH gradient and protecting the underlying cells from the effects of high concentrations of hydrogen ions. Secondly, the reduction in the quantity of acidic groups (such as sialic acid) on mucus which are known to contribute to its protective property of possessing high viscosity [31], may reduce the protective properties of mucus. Thirdly, reduction in the acidic groups may lead to an increase in luminal proteolysis of mucus glycoproteins decreasing the protective value of mucus in preventing injury to mucosal cells [32]. The consequences of inhibition of mucus biosynthesis may, therefore, be important in the pathogenesis of lesion development induced by NSAI drugs.

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REFERENCES

1. R. Menguy and Y. F. Masters, *Surgery Gynec. Obstet.* **120**, 92 (1965).
2. S. Narumi and M. Kanno, *Jap. J. Pharmac.* **22**, 675 (1972).
3. K. D. Rainsford, *Agents and Actions* **5**, 326 (1975).
4. I. P. T. Hakkinen, R. Johansson and M. Pantio, *Gut* **9**, 712 (1968).
5. P. W. Kent and A. Allen, *Biochem. J.* **106**, 645 (1968).
6. J. B. Dekanski, A. MacDonald, P. Sacra and D. V. Parke, *Br. J. Pharmac. Chemother.* **55**, 387 (1975).
7. E. Ezer and L. Szporny, *J. Pharm. Pharmac.* **22**, 143 (1970).
8. T. T. Terho and K. Hartiala, *Biochem. biophys. Acta* **304**, 591 (1973).
9. K. D. Rainsford, *Gut* **16**, 514 (1975).
10. B. E. Lukie and G. G. Forstner, *Biochim. biophys. Acta* **261**, 353 (1972).
11. P. D. Dawkins, B. J. Gould and M. J. H. Smith, *Biochem. J.* **99**, 703 (1966).
12. J. C. Turner, *Int. J. appl. Radiat. Isotopes* **20**, 499 (1969).
13. G. A. Bruno and J. E. Christian, *Analyt. Chem.* **33**, 650 (1961).
14. M. Pfeffer, S. Weinstein, J. Gaylord and L. Indindoli, *Analyt. Biochem.* **39**, 46 (1971).
15. C. Francois, R. D. Marshall and A. Neuberger, *Biochem. J.* **83**, 335 (1962).
16. L. C. Mokrasch, *Analyt. Biochem.* **18**, 64 (1967).
17. K. G. Holden, N. C. F. Yim, L. J. Griggs and J. A. Weisbach, *Biochemistry N.Y.* **10**, 3110 (1971).
18. R. M. Zacharius, T. E. Zell, J. H. Morrison and J. J. Woodlock, *Analyt. Biochem.* **30**, 148 (1969).
19. P. Draper and P. W. Kent, *Biochem. J.*, **86**, 248 (1963).
20. A. H. Wardi and G. A. Michos, *Analyt. Biochem.* **49**, 607 (1972).
21. I. Smith, in *Chromatographic and Electrophoretic Techniques* (Ed. I. Smith), Vol 2. p. 382. Heineman, London (1968).
22. C. A. Pasternak and P. W. Kent, *Research, London*, **5**, 486 (1952).
23. K. D. Rainsford, *Agents and Actions* **5**, 553 (1975).
24. C. H. Morris, J. E. Christian, R. R. Landolt and W. G. Hansen, *J. Pharm. Sci.* **62**, 1017 (1973).
25. H. A. Krebs, *Biochim. biophys. Acta* **4**, 249 (1950).
26. I. D. Capel, M. R. French, P. Millburn, R. L. Smith and R. T. Williams, *Xenobiotica* **2**, 25 (1972).
27. D. Snary and A. Allen, *Biochem. J.* **123**, 845 (1971).
28. N. P. Reed, S. R. Powers and J. A. Balint, *Gastroenterology*, **52**, 1114 (1967).
29. T. M. Brodie, *J. Pharmac. exp. Ther.* **117**, 39 (1956).
30. L. Thomkins and K. H. Lee, *J. Pharm. Sci.* **58**, 102 (1969).
31. A. Gottschalk and M. A. W. Thomas, *Biochim. biophys. Acta* **46**, 91 (1961).
32. G. Prino, A. Lieti and G. Allegra, *Am. J. dig. Dis.* **17**, 863 (1972).