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STUDIES ON THE POSSIBLE ABSORPTION OF A SULPHATED GLYCOPEPTIDE (GLPS) IN RELATION TO ITS MODE OF ACTION

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Abstract—The absorption of an acute dose of glycopeptide sulphate, GLPS, has been studied in free-ranging rats, in rats and dogs after suppression of the gut microflora, in rats with histamine-induced gastric ulcers and in two human subjects, using tracer techniques. Unchanged GLPS is unabsorbed from the mammalian gut and ulcer lesions do not diminish the barrier against the ingress of drug into vascular circulation; differences in faecal excretion rates are readily explicable in terms of the suppression of the gut micro-flora in the antibiotic treated animals and the correspondingly uncomplicated passage of unchanged GLPS through the gut. The intestinal microflora does not bring about systematic degradation of GLPS, and the low proportion of sulphate hydrolysis, which occurs in the lumen of antibiotic treated and untreated animals, and which accounts for the excretion from the body of SO_4^{2-} and small organic sulphates, is compatible with that produced, for example, by dialysis *in vitro*. Available evidence suggests that GLPS may inhibit the inflammatory process by exerting a stabilizing effect on the cell membrane and adjacent small vessels.

A GLYCOPEPTIDE, mol. wt 81,000, called GLP, which contains hexoses, hexosamines, sialic acid and a polypeptide residue,[1] and which possesses anti-inflammatory and anti-ulcerogenic activities in laboratory animals, has been isolated from pig-duodenum constituents and characterized at Crinos Biological Research Laboratories, Como, Italy. The polysaccharide residue appears to be retained by the chemical sulphation product, GLPS (approximately 12% S),[2] which possesses enhanced pharmacological properties.[3] Not only has GLPS anti-ulcerogenic activity superior to GLP,[3,4] but also anti-peptic properties, especially in gastric juice.[5,6]

Despite the accepted use of GLPS in gastroenterology,[7] the mechanism of its anti-inflammatory and anti-ulcerogenic action is obscure. Knowledge about whether the drug is absorbed from the gastro-intestinal tract is essential to an understanding of its mode of action, particularly since pharmacological activity follows oral administration. The present work was designed to investigate gastro-intestinal absorption of GLPS in relation to its anti-inflammatory and anti-ulcerogenic properties.

MATERIALS AND METHODS

Preparative methods. Sulphation[2] of a small batch of finely-divided glycopeptide (2·1 g), which was kindly provided by Crinos Biological Research Laboratories, afforded 2·7 g of GLPS (Found: N, 2·8; S, 12·4; hexoses, 11·4; hexosamines, 19·4; sialic acid, 2·4%), which gave electrophoretic mobilities and colour reactions, identical with authentic material (Found: N, 2·8; S, 11·9; hexoses, 12·0; hexosamines, 20·2; sialic acid, 2·5%), also provided by Crinos Biological Research Laboratories.

Repetition of the sulphation procedure[2] with the same batch size of glycopeptide,

but this time using 116 mg of chloro[^{35}S]sulphonic acid (28·6 mCi/mmole) and the remainder of the sulphating agent as unlabelled chlorosulphonic acid (ca. 10·5 g), yielded approximately 2·7 g of [^{35}S]GLPS with a sp. activity of 1·169 μCi/mg.

[^{35}S]GLPS and unlabelled GLPS both had identical electrophoretic mobilities on cellulose acetate paper, impregnated in borate buffer, pH 8·7 (300 ml of 0·05 M-tetraborate and 200 ml of 0·2 M-boric acid diluted to 1 l. with water), at an applied voltage of 5·3 V/cm. The zones stained identically with an 0·06 per cent solution of Toluidine Blue in 0·5% (v/v) acetic acid. Autoradiography of the electrophoretograms in conjunction with densitometry on the resulting photographic plates showed that at least 97 per cent of the ^{35}S was associated with the GLPS zone.

Experiments with animals. Adult rats (150–200 g body wt) were used (CFY strain from Carworth Europe, Huntingdon, England).

For the metabolism studies, 12 rats, six of each sex, were kept individually in glass metabolism cages.[8] In these experiments, 1·0 ml of an aqueous solution of [^{35}S]GLPS (30 mg) was administered to each animal by stomach tube. Food and water were supplied *ad lib.* and the urine and faeces were collected daily and stored at −20°; the urine being collected in vessels surrounded by Dewar flasks containing cardice. After 5 days, the rats were killed by asphyxiation with CO_2, and the carcass and remaining viscera, after removal of the entire gastro-intestinal tract, were stored at −20°.

A second experiment was made with six rats, in which the microflora of the gut had previously been suppressed by the daily administration of oxytetracycline (100 mg) plus succinylsulphathiazole (500 mg) to fasting animals, which were allowed access only to glucose–saline during the treatment. Faecal samples from these animals, compared with controls, showed that the bacteria had almost been entirely eliminated after 24 hr treatment with the antibiotics. These animals were then treated orally with [^{35}S]GLPS and placed in metabolism cages for collection of the urine and faeces, as described previously.

A third experiment was made with six rats with experimentally induced gastric ulcers, and in which the microflora of the gut had also been previously suppressed with antibiotics. To fasting rats, which were maintained on glucose–saline at pH 2·0, a subcutaneous dose of histamine phosphate (16 mg) was administered to each of the animals on 2 consecutive days. Four animals, which were then examined, showed widespread small, sub-mucosal ulcers in the pyloric region of the stomach as well as occasional craterous ulcers in the fundal region. After ulceration had been established, each rat was dosed with oxytetracycline plus succinylsulphathiazole to suppress the gut flora in the same way as in the second experiment. These rats were then treated orally with [^{35}S]GLPS and placed in metabolism cages for collection of the urine and faeces, as described previously.

Adult beagles (10 kg body wt) were used. The animals were kept under close observation for 7 days before experimentation, during which time they were checked for general physical well-being, and neomycin sulphate (3 g) was administered daily. Faecal samples from these animals, compared with controls, revealed that the microflora of the gut had been suppressed by 1 week's treatment to the extent of 20 per cent of that of the controls. Further reduction in the gut flora was considered to be detrimental to the normal nutritional status of the dog. After placing in the metabolism cages, each of the dogs was dosed orally with a single gelatin capsule containing (150 mg) of [^{35}S]GLPS. Unrestricted food and water were supplied. Urine and faeces

were collected separately, at daily intervals, for 5 days; urine and faeces samples were stored at −20°.

Human volunteers. Permission was obtained from the Medical Research Council's Advisory Panel on the Allocation of Radioactive Isotopes for Clinical Use, for the administration of 30 μCi of [^{35}S]GLPS to two adult male subjects. With their informed consent, two adult male volunteers were selected for participation in this investigation, to whom a clinical examination had been given and shown them to be in good health, with normal hepatic and renal functions. Both subjects fasted for 14 hr before the administration of encapsulated [^{35}S]GLPS powder. Subject A, of body wt 86 kg, received 121·1 mg (29·2 μCi) of [^{35}S]GLPS, and Subject B, of body wt 61 kg, received 124·6 mg (30·1 μCi) of [^{35}S]GLPS. Urine and faeces were collected daily, and stored at −20° for analysis. Throughout the period of this investigation, both volunteers were under strict medical supervision. There were no adverse reactions.

Mucosal binding of GLPS. Twelve rats, which had previously been fasting for 18 hr, were administered [^{35}S]GLPS (25–33 mg) p.o., and killed by asphyxiation with CO_2 at various times thereafter. The stomach was ligated at the fundal end and the duodenum at a point about 6 cm below the pylorus. The stomach and attached duodenal segments were opened longitudinally, and gently washed 4 times in distilled water. The organs were pinned out, and the mucosa was gently scraped from the underlying tissue with a scalpel blade. Yields of mucosal cells from the fundal part of the stomach were low, those from the pyloric region were intermediate and those from the duodenum were relatively high. Mucosal scrapings, 400 ± 100 mg wet wt, were placed in a tared counting vial, 1·15% (w/v) KCl (6 ml) was added, and the mixture was shaken. Samples of this mixture (2 × 2 ml) were counted for ^{35}S after digestion with NCS solubilizer (0·5 ml) for 2 hr at 37° and addition of the toluene-Triton X-100 scintillator system (10 ml).[9] A further 2 ml sample of the mixture was submitted to equilibrium dialysis[10] against 1·15% (w/v) KCl (2 ml) for 12–24 hr. The gastric-duodenal washings and diffusate were also counted for ^{35}S.

Subcellular binding of GLPS. The stomach and duodenum of 7 treated rats were processed *v. supra*, but the tissues were homogenized in 1·15% (w/v) KCl (20 ml) with an Ultra-Turrax homogenizer. A sample of the whole homogenate was counted for ^{35}S, and the remainder was differentially centrifuged in a Mark II, MSE Superspeed 65 Ultracentrifuge (Measuring & Scientific Equipment Limited, London, S.W.1) employing Rotor No. 59596. The nuclear fraction was obtained by centrifugation at 1000 *g* for 20 min, the mitochondrial fraction at 10,000 *g* for 20 min, and the microsomal fraction at 50,000 *g* for 2 hr. In each case, the supernatant was counted for ^{35}S.

Measurement of radioactivity. A Mark 1 Liquid Scintillation Counter (Nuclear Chicago Corporation, Des Plaines, Ill., U.S.A.) was used for measurement of ^{35}S using standard channels, ratio quench correction curves. For the measurement of ^{35}S in urine and in solvent extracts of faeces, portions were mixed with the toluene-Triton X-100 based scintillator.[9] Portions of the residue from the solvent extraction of faeces were digested in concentrated HNO_3 to convert ^{35}S to $^{35}SO_4^{2-}$, and then samples of the diluted digest were neutralized with Tris and mixed with the toluene-Triton X-100 scintillator[9] for liquid scintillation counting as above. Rat carcasses were cut into small portions when frozen and passed through an electric mincing machine, cooled with solid CO_2. The resulting powder was lyophilized, and the samples digested, as previously, for measurement of ^{35}S.

RESULTS

The excretion of ^{35}S from normal rats treated with [^{35}S]GLPS is shown in Table 1. It is evident that almost all of the radioactivity resulting from a single oral dose had been excreted in the faeces within 5 days. Excretion of ^{35}S in the 5-day collection of urine did not exceed 0·5 per cent of the dose in any one of the twelve animals examined. The small urinary excretion of ^{35}S was less likely to be due to gastro-intestinal absorption of the intact [^{35}S]GLPS than to liberation from [^{35}S]GLPS in the alimentary canal of a low molecular-weight [^{35}S] intermediate, followed by its absorption. Retention of ^{35}S by the animals was correspondingly negligible, and in none of the animals investigated was more than 0·1 per cent of the dose detected in the intestinal contents at the end of the experiment (Table 1).

TABLE 1. EXCRETION AND RETENTION OF RADIOACTIVITY IN NORMAL RATS AFTER ORAL ADMINISTRATION OF [^{35}S]GLPS

Animals	Recovery ^{35}S during 5-day collection (% of dose)				
	Faeces	Urine	Intestinal residue	Carcass plus viscera	Total
Males* Mean	88·8	0·5	0·03	<0·01	89·3
±S.E.M.	5·4	0·03	0·007		5·4
No. of independent observations	6	6	6	6	6
Females* Mean	86·1	0·5	0·03	<0·01	86·6
±S.E.M.	3·8	0·03	0·006		3·9
No. of independent observations	6	6	6	6	6

* Dose of 30 mg.

The radioactivity was almost completely eliminated in the 3-day faeces of normal rats treated with [^{35}S]GLPS. Male rats excreted 76·9 ± 4·7 per cent of the dose by the faecal route in 24 hr, but the females only 22·9 ± 9·5 per cent. However, after 3 days, the male rats had excreted 88·7 ± 5·4 per cent of the dose in their faeces and the females 86·1 ± 4·2 per cent.

The non-absorption[11,12] of [^{35}S]GLPS from the gut was confirmed in rats equipped with biliary fistulae. The amount of ^{35}S secreted into the bile was very low, and was 10-fold less than that found in the urine of rats, employed in the excretion–retention study (Table 1).

Equilibrium dialysis of the pooled urine from six treated rats against 0·1 M-phosphate buffer, pH 7·4, resulted in the compartmentalization of 49 per cent of the available ^{35}S on both sides of the dialysis membrane. Since it had previously been found that in the case of a readily diffusible compound, equilibrium was established under these conditions within 3 hr, it follows that 98 per cent of the radioactivity excreted in the urine of those rats was readily diffusible and could not therefore have been due to the non-diffusible [^{35}S]GLPS *per se*.

When samples of pooled urine from the treated rats were incubated at 37° for 24 hr with aryl sulphatase preparations, and the resulting media treated with barium

hydroxide, 75 per cent of the ^{35}S originally present in the urine corresponded demonstrably to $^{35}SO_4^{2-}$. However, a small proportion of the ^{35}S in the urine was possibly due to diffusible organic sulphate, unhydrolysed by sulphatase preparations. Our experiments do not permit any conclusion to be made about the nature of this (these) small [^{35}S] molecule(s).

The faecal excretion of ^{35}S from the rats fed antibiotics and [^{35}S]GLPS is shown in Table 2. Almost all of the label had been excreted in the faeces at the end of 5 days. Differences in faecal excretion rates between these animals and those in the previous experiment were due to the suppression of the gut microflora in the antibiotic treated animals and the correspondingly uncomplicated passage of unchanged GLPS through the gut.

TABLE 2. EXCRETION OF RADIOACTIVITY FROM RATS IN WHICH THE MICROFLORA OF THE INTESTINE HAD BEEN SUPPRESSED BY TREATMENT WITH ANTIBIOTICS BEFORE ORAL ADMINISTRATION* OF [^{35}S]GLPS

	Recovery ^{35}S during 5-day collection (% of dose)			
Animals	Faeces	Urine	Intestinal residue	Total
Mean	96·0	0·7	<0·01	96·7
±S.E.M.	1·2	0·2		1·1
No. of independent observations	6	6	6	6

* Dose of 30 mg.

If the excretion of ^{35}S in normal rats (see Table 1) is compared with the excretion of ^{35}S in those in which ulcers were experimentally-induced by successive subcutaneous doses of histamine (see Table 3), it will be seen that in both cases the excretion of the label was essentially complete after 5 days, and that almost exactly the same proportion of the label was excreted by the faecal route irrespective of the absence or presence of gastric ulcers. It ought to be stipulated that the control animals with gastric ulcers, which were sacrificed after 2 days of histamine treatment, exhibited an average ulcer score which was rather higher than the histamine-treated rats which were subsequently given antibiotics prior to [^{35}S]GLPS (Table 3). Some evidence for wound healing was therefore observed, which may have been a result of subsequent treatment with antibiotics, or of a return to *ad lib.* feeding and the discontinuance of histamine administration.

The excretion of ^{35}S from healthy Beagles, in which the gut micro-flora had been partially suppressed by antibiotics, shows that virtually all of the radioactivity from a single oral dose of [^{35}S]GLPS had been excreted in the faeces in the 5 days after administration (Table 4). Excretion of ^{35}S in the 5-day urine collection did not exceed 0·6 per cent of the dose in any of the animals employed. These data are consistent with those for rats, treated with antibiotics (cf. Table 2).

Finally, the results of an excretion experiment in two male human subjects showed that the radioactivity from a single oral dose of [^{35}S]GLPS (120–125 mg) was rapidly eliminated from the body. After 4 days, subject A had excreted 85·3 per cent of the

TABLE 3. EXCRETION OF RADIOACTIVITY FROM RATS WITH HISTAMINE-INDUCED GASTRIC ULCERS IN WHICH THE MICROFLORA OF THE INTESTINE HAD BEEN SUPPRESSED BY TREATMENT WITH ANTIOBIOTICS BEFORE ORAL ADMINISTRATION* OF [^{35}S]GLPS

	Recovery ^{35}S during 5-day collection (% of dose)			
Animals	Faeces	Urine	Intestinal residue	Total
Mean	89·0	1·3	< 0·01	90·3
±S.E.M.	1·6	0·3		1·5
No. of independent observations	6	6	6	6

* Dose of 30 mg.

TABLE 4. EXCRETION OF RADIOACTIVITY FROM BEAGLES IN WHICH THE MICROFLORA OF THE INTESTINE HAD BEEN PARTIALLY SUPPRESSED BY TREATMENT WITH NEOMYCIN* BEFORE ORAL ADMINISTRATION† OF [^{35}S]GLPS

	Recovery ^{35}S during 5-day collection (% of dose)		
Animals	Faeces	Urine	Total
Dog 1♂	96·5	0·6	97·1
Dog 2♂	88·9	0·4	89·3
Dog 3♀	101·8	0·3	102·1
		Mean	96·2

* 3 g of Neomycin sulphate was administered daily for 1 week (for details, see Materials and Methods).
† 150 mg, in capsule form.

dose in the faeces, 10·3 per cent after 2 days, and the corresponding values for subject B were respectively 94·9 and 19·5 per cent; both subjects were constipated on certain days. The individual recoveries of administered radioactivity are remarkably satisfactory (see Table 5), especially if the bulk of the faeces, which had to be homogenized, is taken into consideration. Up to 1 per cent of the dose was excreted in the urine, and again, this was unassociated with GLPS.

Substantial amounts of ^{35}S are adsorbed on to gastric-duodenal mucosal cells with a half-life of approximately 1 hr and a few microgrammes are held there for longer periods of time (Fig. 1). The log–log relationship between the amount absorbed and time, over a 10,000-fold range of concentration, is reminiscent of the graphical representation of Freundlich's adsorption isotherm (Fig. 1). ^{35}S could not be detected in association with the mucosa after 96 hr; the limits of detection were about 0·2 μg/g of mucosa. Since the ^{35}S bound to the mucosa was not diffusible, it was probably

TABLE 5. EXCRETION OF RADIOACTIVITY FROM HUMAN SUBJECTS DOSED ORALLY WITH [^{35}S]GLPS

	Recovery of administered radioactivity (% of the dose)		
	Urine*	Faeces†	Total
Subject A	1·0	95·4	96·4
Subject B	0·9	95·9	96·8

* Collected for 5 days.
† Collected for 7 days.

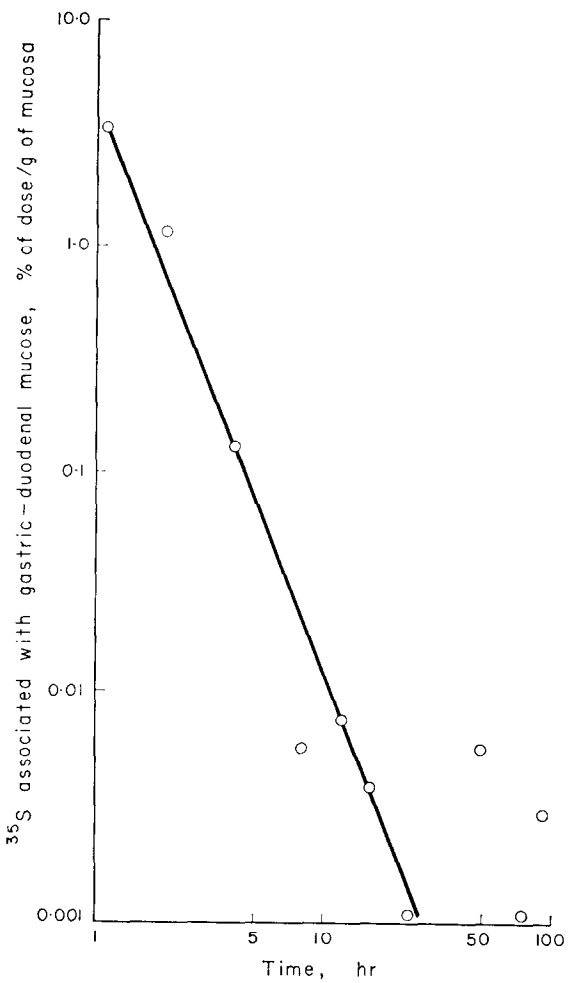

FIG. 1. Log–log relationship between the amount of [^{35}S]GLPS adsorbed by rat stomach, duodenum mucosa and time. An oral dose (25–33 mg) was given to each of 12 rats (for details, see Materials and Methods).

associated with GLPS. In preliminary experiments in rats, variations in the stomach and duodenum ^{35}S contents, 1 and 2 hr after dosing, were ascribed to differences in pyloric opening.

An investigation of the sub-cellular distribution of ^{35}S in the rat stomach and duodenum (Table 6) showed unspecific binding of GLPS, commensurate with adsorption to the cell membrane. In that case, desorption of GLPS would have occurred through homogenization of the cells with general binding to all of the liberated organelles and membrane structures. On centrifugation, ^{35}S was, in fact, proportionally distributed amongst each precipitated fraction.

TABLE 6. LOCATION OF ^{35}S AMONGST THE SUB-CELLULAR COMPONENTS OF RAT STOMACH AND DUODENUM†

Time after dosing* (hr)	Radioactivity remaining in the gastric–duodenal contents (% of dose)	Sub-cellular fraction (% dose/g of tissue)			
		Nuclear‡	Mitochondrial	Microsomal	Cytosol
1	24·97	0·36	0·01	0·02	0·01
2	0·36	0·15	0·01	0·01	0·01
4	0·28	0·015	0·002	0·002	0·002
8	0·07	0·001	0·001	§	§
12	0·03	0·002	§		
16	0·48	0·005	0·001		
24	0	0·002	§		
72	0	§			
96	0				

* A single oral dose of 25–33 mg to each of 7 rats.
† For details, see Materials and Methods.
‡ This fraction contained cell membranes, nuclei and cell debris.
§ Denotes below the limits of detection.

DISCUSSION

These data provide direct evidence that unchanged GLPS is unabsorbed from the gastro-intestinal tracts of rats and dogs and of man after administration of the drug p.o. Almost all of the radioactivity, when [^{35}S]GLPS was employed, was recovered in the faeces and negligible amounts were in the urine.

Three comments appear to be relevant. Firstly, because the drug was unabsorbed by rats in which extensive gastric ulceration had been induced by histamine treatment, it would appear that these lesions do not diminish the barrier properties of the gastro-intestinal system against the entry of GLPS into systemic circulation. Secondly, since very little ^{35}S is excreted into the urine of animals after ingestion of GLPS and irrespective of their possible treatment with antibiotics, it follows that relatively protected amino- and hydroxyl groups of glycopeptide were in fact sulphated. The very low concentration of ^{35}S, which is excreted via the kidneys of animals after treatment with GLPS is comparable with that resulting from similar treatment with amylopectin-[^{35}S]sulphate.[13] Thirdly, the drug appeared to be bound to the gastric and duodenal

mucosa for several hours, but this was clearly a reversible adsorption effect, which was utterly independent of vascular circulation, since GLPS was unabsorbed.

It is noteworthy that sodium amylopectin[13] and chondroitin sulphate[14,15] are unabsorbed from the mammalian gut, but there is conflicting evidence about the possible absorption of certain other high molecular weight natural products, for example heparin.[16,17]

The non-absorption of GLPS is incommensurate with mammalian metabolism *per se*, and the present work indicates that systematic degradation of GLPS by the gastro-intestinal microflora does not occur. Thus, although sulphate hydrolysis of GLPS with accompanying sulphation of small organic molecules occurs to a very limited extent in the lumen, and the resulting molecular species are absorbed from the gut and excreted from the body via the bile and urine, the same low degree of sulphate hydrolysis is compatible, for example, with dialysis *in vitro*.

In general, the anti-inflammatory properties of drugs have been associated with some gastroenterological damage, suggesting that anti-inflammatory agents share a fundamental mode of action, which accounts for both their desirable and undesirable effects. Thus, phenylbutazone and salicylates inhibit mucopolysaccharide synthesis and possess anti-myotic properties.[18,19] The available evidence seems to indicate that GLPS inhibits the inflammatory process with a different mechanism, possibly by exerting a stabilizing effect on the cell membrane and adjacent small vessels.[20–22] Relevant to this idea, and in the context of gastric inflammation, are (1) the non-absorption of GLPS, (2) its capacity for solvolysis and adherence to membranes and, (3) the adsorption characteristics of the gastric mucosal cells resulting in the adsorption from aqueous solution of substantial amounts of drug with a short half-life and of a few microgrammes for much longer periods of time. Further evidence is required for this supposition, and it is not meant to imply that GLPS acts only in this way. Whether such a mechanism would account for both the anti-inflammatory and anti-ulcerogenic properties of the drug is unknown. GLPS may interfere specifically with gastric secretion, and in that connexion, anti-peptic properties have been reported already.[4–6]

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