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Biosynthesis of Intestinal Mucins

2. INCORPORATION OF [^{35}S]SULPHATE BY GUINEA-PIG COLON *IN VITRO**

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In a previous paper (Pasternak, Kent & Davies, 1958) the uptake of [^{35}S]sulphate by isolated gastrointestinal tissues of a number of animals was surveyed; the highest incorporation was found to occur in guinea-pig colon. This tissue was accordingly chosen for a more detailed investigation of fixation of [^{35}S]sulphate. A brief account of part of this work has already been presented (Pasternak, Kent & Davies, 1956).

METHODS

Preparation of tissue. Tissue was prepared as described by Pasternak *et al.* (1958).

Incubation. The incubation medium was that described by Pasternak *et al.* (1958), glucose being omitted. Substrates were added to yield final concentrations of 0·02 M, and incubated with tissue for 3 hr. except where otherwise stated; sodium pyruvate was kindly given by Mr R. W. Wakelin. Other details of incubation were as described by Pasternak *et al.* (1958).

Extraction of tissues. Tissues after incubation were extracted with 5 ml. of NaOH (2%, w/v) for 12 hr. at room temperature. The resulting extracts were centrifuged to remove residual cell fragments before dialysis. This treatment did not result in significant loss of bound sulphate by hydrolysis.

Dialysis. Dialysis of extruded mucin (i.e. that which passes into the incubation medium) and extracted mucin (i.e. mucin obtained from the tissue after incubation) (as defined by Pasternak *et al.* 1958) was carried out in cellophan against running tap-water (16°), constant activity being reached within 90 hr. Carrier Na_2SO_4 and penicillin plus streptomycin were added every 12 hr. as described by Pasternak *et al.* (1958).

* Part 1: Pasternak, Kent & Davies (1958).

Electrophoresis. The method of Kunkel & Tiselius (1951) was employed, sodium diethylbarbiturate buffer (0·07 M, pH 8·8) and Whatman no. 1 paper (8 cm. × 32 cm.) being used. Three mucin samples (0·025–0·1 ml.) were investigated simultaneously; by using three paper strips and four glass plates 'sandwich-wise' up to nine samples could be included in one run. At the end of electrophoresis (6 hr., 120 v; 4·3 v/cm.) the paper was dried and cut into longitudinal strips, each strip containing the separated components of one sample.

Dry-weight determination. Dry weights of tissues were determined as described by Pasternak *et al.* (1958).

Radioactive assay. Samples of the mucin solutions before and after dialysis were assayed as described by Pasternak *et al.* (1958). Alternatively, the proportion of added isotope in the [^{35}S]mucin was obtained by paper electrophoresis of a sample (0·05–0·08 ml.) of the medium or tissue extract after incubations.

Paper strips after electrophoresis were assayed (GM mica end-window tube EHM2s operating at 1640 v) as contiguous rectangles (1·7 cm. × 1 cm.) along the length of the strip. The radioactive peak at the origin was identified as [^{35}S]mucin, whereas that which had migrated towards the anode was shown to be free sulphate (approx. $1{\cdot}4 \times 10^{-2}$ cm./v/hr.). The term [^{35}S]mucin is used throughout this paper to denote ^{35}S-labelled, [^{35}S]sulphate-free extruded or extracted mucin. It was considered unnecessary to correct for self-absorption, since the thickness of the paper, which is decisive for this isotope, is the same for both radioactive components. The radioactivities of the two components were determined by summing the counts below each of the plotted peaks and correcting for background (approx. 10 counts/min./cm.). The proportion of added isotope found in the [^{35}S]mucin was calculated from the ratio {[^{35}S]mucin (counts/min.) × 10^5}:{[^{35}S]sulphate + [^{35}S]mucin (counts/min.)} and expressed as counts/min./100 000 counts/min. initially added/mg. of tissue dry wt. (subsequently abbreviated as counts/mg. of tissue). [^{35}S]Mucin (counts/min.) represents the total counts given

by a mucin band on an electrophoretic strip, and [^{35}S]-sulphate the counts of the sulphate band on the same strip.

Amino sugar content and penetration by [^{35}S]*sulphate.* These were determined as described by Pasternak *et al.* (1958).

RESULTS

Determination of [^{35}S]*mucin.* In the early part of this investigation the amount of [^{35}S]sulphate incorporated during incubation was determined on measured samples of mucin which had been dialysed against distilled water for 90–130 hr. (Pasternak *et al.* 1958) or against running tap-water for 90 hr. The dialysis procedure, however, is not completely satisfactory; Bostrom & Mansson (1953) were unable to remove free sulphate completely from extracts of cartilage by dialysis, and therefore used a method of separation on Dowex 2 columns. We have not encountered this difficulty (see Table 2). On the other hand, another disadvantage of the dialysis procedure is the resulting change in volume of mucin solution. Such volume changes make calculation of total [^{35}S]mucin difficult. We have therefore employed paper electrophoresis between glass plates for the separation of free sulphate and incorporated sulphate. The advantage of this method as an analytical tool is that only a small portion of mucin solution ($<0{\cdot}1$ ml.) is required and that several determinations can be made within a day (cf. 90 hr. for complete dialysis). On the other hand, the counting efficiency in assaying [^{35}S]mucin or [^{35}S]sulphate on paper (electrophoretic procedure) is only 25% of that on Perspex disks (dialysis procedure).

In order to isolate [^{35}S]mucin remaining after a portion of solution had been used for electrophoretic assay, dialysis, as described above, followed by freeze-drying, was employed.

A comparison of the results of dialysis and electrophoresis in determining incorporated [^{35}S]-sulphate is given in Table 1. The agreement in percentage counts incorporated (from which counts/mg. of tissue are calculated) indicates that non-dialysable ^{35}S may be equated with electrophoretically immobile ^{35}S, and provides evidence for the reality of the incorporation process.

Values of incorporated ^{35}S quoted subsequently were obtained either by dialysis (provided that large volume changes did not occur) or by electrophoresis. Each experiment contained an internal control (tissue incubated for 3 hr. without addition of substrate). The values of sulphate incorporation in Table 3 are calculated as ratios of this control.

Hydrolysis of [^{35}S]*mucin.* A sample of ^{35}S-labelled mucin was hydrolysed (2 hr., 4N-HCl at 110°), and the solution was evaporated *in vacuo* over KOH and examined by paper chromatography (M-ammonium acetate–ethanol, 2:5, v/v) on Whatman no 1 paper. Included on the chromatogram were samples of unhydrolysed mucin and the following amino acids: methionine, cystine, taurine and cysteic acid. Fig. 1 shows that at least 90%

Table 1. *Determination of* [^{35}S]*mucin by dialysis and electrophoresis*

Values (in counts/min.) refer to the extruded mucin fraction obtained during a typical experiment. Experimental details and corrections are described under Methods. Incorporation is the percentage of the added counts in the mucin after incubation. Calculated from paper-electrophoretic separations.

Dialysis		
Non-dialysable counts (0·2 ml.)	Total counts (0·05 ml.)	Incorporation (%)
4656	43 680	2·7
Electrophoresis		
Immobile counts (0·1 ml.)	Total counts (0·1 ml.)	Incorporation (%)
501	18 300	2·7

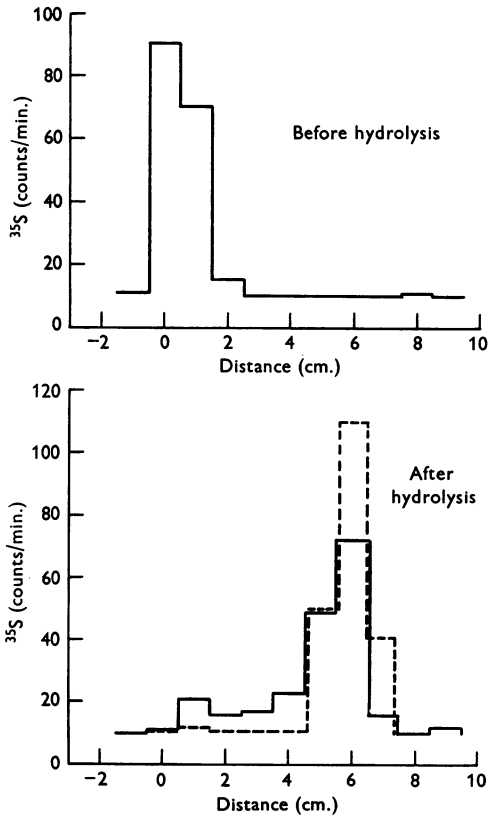

Fig. 1. Chromatographic analysis (ethanol–ammonium acetate) of [^{35}S]mucin (continuous line) before and after hydrolysis with 4N-HCl at 110° for 2 hr. [^{35}S]Sulphate marker is shown as a broken line.

of the original radioactivity can be recovered as free sulphate. The R_F of sulphate in this system is 0·23, and that of the other compounds is: cysteic acid 0·26, cystine 0·36, taurine 0·60 and methionine 0·70.

Effect of time on incorporation of [^{35}S]*sulphate.* In order to examine the fixation of [^{35}S]sulphate more closely, segments of colon were incubated for varying periods of time. Fig. 2 shows the result of such an incubation. The rate of appearance of [^{35}S]mucin fractions begins to fall in both fractions after 1 hr.; this decrease is accompanied by a decrease in oxygen uptake. (Reduction of Q_{O_2} is less marked in the presence of glucose.) Decrease in rate of uptake of [^{35}S]sulphate with time was also observed in an experiment in which tissues were pre-incubated in the absence of [^{35}S]sulphate for varying periods up to 2 hr. At the end of these times [^{35}S]sulphate was added and tissues were incubated for a further 3 hr. Tissues which had been pre-incubated longest fixed the least [^{35}S]sulphate.

Effect of various treatments. The results are summarized in Table 2; incorporation of [^{35}S]-sulphate by extracted mucin was similar to that by the extruded fraction. Incubation in nitrogen abolished uptake of [^{35}S]sulphate almost completely. This suggests that the incorporation process is an endergonic one, a view supported by the effect of certain inhibitors (Table 3). Heating the tissue to 100° or freezing it in liquid air before incubation abolished uptake of [^{35}S]sulphate completely.

The fourth treatment cited in Table 2 consisted of incubating tissue without [^{35}S]sulphate for 2 hr. The tissue was then removed and [^{35}S]sulphate added to the medium, which now contained extruded mucin; this was re-incubated for a further 3 hr. No radioactivity was found in the extruded fraction at the end of dialysis. This experiment shows that preformed mucin in the absence of tissue does not incorporate [^{35}S]sulphate; moreover, it provides a useful control for the dialysis procedure, since it demonstrates that free [^{35}S]sulphate is completely removed in the presence of unlabelled mucin. Lack of uptake of ^{35}S without tissue suggests that the necessary enzymes are intracellular and are not extruded with mucin.

A further experiment indicates that sulphatases are not present in extruded mucin; tissue was incubated in medium containing [^{35}S]mucin instead of [^{35}S]sulphate: no dialysable ^{35}S-labelled products were formed, nor was there any penetration of tissue by [^{35}S]mucin; reabsorption of mucin (albeit a dialysed and freeze-dried product) does not therefore appear to occur *in vitro*. The behaviour of homogenized tissue towards [^{35}S]sulphate has also been studied and will be discussed later.

Effect of added substrates. The effect of added substrates on oxygen uptake, penetration of [^{35}S]-sulphate and incorporation of [^{35}S]sulphate by extruded mucin is shown in Table 3; similar results were obtained with extracted mucin. The substrates which cause significant stimulation are D-glucose (Q_{O_2}, penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate), sodium pyruvate (Q_{O_2} and [^{35}S]sulphate incorporation) and D-glucosamine ([^{35}S]sulphate incorporation).

Sodium fluoroacetate, phenol and salicylic acid (all at 20 mM) are inhibitory. Since these substances

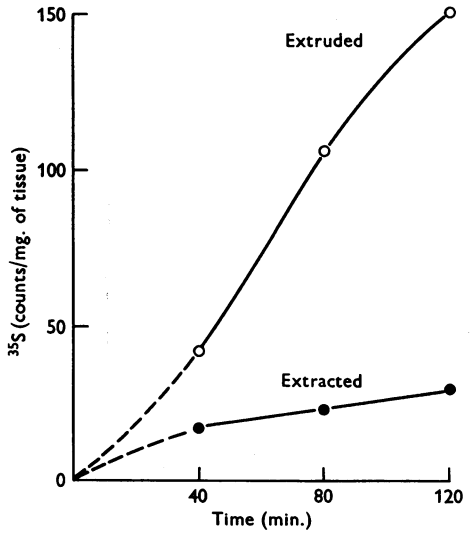

Fig. 2. Incorporation of [^{35}S]sulphate into extruded (○) and extracted (●) mucin factions by sections of guinea-pig colon incubated at 37° in Krebs–Ringer buffer.

Table 2. *Effect of various treatments on* Q_{O_2} *and incorporation of* [^{35}S]*sulphate*

Some values are averages derived from several experiments, the number performed being given in parentheses. Experimental details are described under Methods and Results.

Treatment	Q_{O_2} (at 150 min.)	Incorporation of [^{35}S]sulphate into extruded mucin fraction (counts/100 000 added/mg. of dry tissue after incubation for 180 min.)
Control	−4·6 (6)	14·1 (6)
Incubation in N_2	—	0·5 (3)
Heating tissue	0·0 (1)	0·0 (1)
Freezing tissue	−1·4 (1)	0·0 (1)
Removal of tissue from medium	0·0 (1)	0·0 (1)
Incubation with [^{35}S]mucin	−5·2 (4)	* (4)

* No [^{35}S]sulphate in extruded fraction; no [^{35}S]sulphate or [^{35}S]mucin in extracted fraction.

Table 3. *Effect of various substrates on Q_{O_2} penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate*

Relative Q_{O_2} values, penetration by ^{35}S and incorporation of ^{35}S are calculated from the ratio: value with substrate/value without substrate. Most values are averages derived from several experiments, the number performed being given in parentheses. Experimental details are described in Methods. (In a preliminary communication (Pasternak, *et al.* 1956) glucosamine was stated to be without effect on uptake of [^{35}S]sulphate; subsequent electrophoretic measurements have shown this to be incorrect.)

Substrate	Relative Q_{O_2} (at 150 min.)	Relative penetration by ^{35}S (after incubation for 180 min.)	Relative incorporation of ^{35}S into extruded mucin fraction (after incubation for 180 min.)
D-Glucose	1·5 (11)	1·4 (3)	4·0 (9)
D-Glucosamine	1·0 (5)	1·0 (2)	1·8 (5)
D-Galactosamine	1·1 (3)	1·0 (2)	0·7 (3)
N-Acetyl-D-glucosamine	0·9 (3)	1·0 (2)	1·0 (3)
Sodium pyruvate	1·6 (5)	1·0 (3)	1·5 (5)
Sodium α-oxoglutarate	0·9 (4)	1·0 (1)	0·7 (4)
L-Glutamine	0·7 (2)	1·0 (2)	0·6 (2)
Potassium ethyl sulphate	0·9 (1)	—	1·0 (1)
Potassium methyl sulphonate	1·3 (1)	—	1·2 (2)
Sodium fluoroacetate (20 mM)	0·5 (1)	—	0·3 (1)
Phenol (20 mM)	0·2 (1)	—	0·0 (1)
Salicylic acid (20 mM)	0·0 (1)	—	0·0 (1)
Salicylic acid (0·5–5 mM)	1·0 (3)	—	0·8 (3)
2:4-Dinitrophenol (0·01–0·1 mM)	0·7 (6)	—	0·6–0·7 (6)

Table 4. *Amino sugar content of mucin fractions*

All values (μg./mg. of dry tissue) are averages of duplicate determinations. Experimental details are described in Methods and Results.

	Extruded mucin (during 30 min. preincubation)	Extruded mucin (during incubation for 120 min.)	Extracted mucin (after incubation for 150 min.)	Total mucin
Control	4·3 (2)	3·4 (2)	7·3 (2)	15·0
With glucose	4·3 (2)	3·7 (2)	7·2 (2)	15·2

abolish respiration almost completely it seems likely that their effect on uptake of [^{35}S]sulphate is due to a suppression of the energy supply (cf. Table 2). Lower concentrations of salicylic acid (0·5–5 mM) are without effect on incorporation of [^{35}S]sulphate, in contrast with the findings of Bostrom & Mansson (1953) and Loewi & Kent (1957); stimulation of respiration by these concentrations of salicylic acid was not observed (cf. the 'uncoupling' action of salicylic acid reported by Smith & Jeffrey, 1956).

Inhibition by low concentrations of 2:4-dinitrophenol (0·01–0·1 mM) is not very marked, and was not obtained without simultaneous depression of Q_{O_2}; 'uncoupling' of oxygen consumption and uptake of [^{35}S]sulphate by this substance has been shown by Boyd & Neumann (1954) and by Kodicek & Loewi (1955).

Sodium α-oxoglutarate, in contrast with sodium pyruvate, does not stimulate but rather depresses Q_{O_2} and incorporation of [^{35}S]sulphate; such an effect was also observed by Loewi (personal communication), working with guinea-pig granulation tissue.

The total amino sugar, which may be taken as a measure of total mucin, is unaffected by addition of glucose (Table 4). In this experiment tissues were incubated for 30 min. before being transferred to fresh vessels, in order to remove as much preformed mucin as possible (Pasternak *et al.* 1958). No stimulation of mucin production was apparent in any of the fractions. The time course of mucin extrusion during this experiment is shown in Fig. 3; the initial sharp rise represents extrusion of preformed mucin, which remains in spite of preincubation. Although the values in Fig. 3 are suggestive of an increase in total amino sugar during incubation, determinations could not in fact be made with sufficient accuracy to warrant such a conclusion. It is therefore not possible to say whether formation of [^{35}S]mucin represents a net increase of mucin, or whether there is an equivalent breakdown of unlabelled mucin during incubation.

DISCUSSION

The evidence which has been presented indicates that the mechanism by which [^{35}S]sulphate becomes incorporated into mucin of guinea-pig

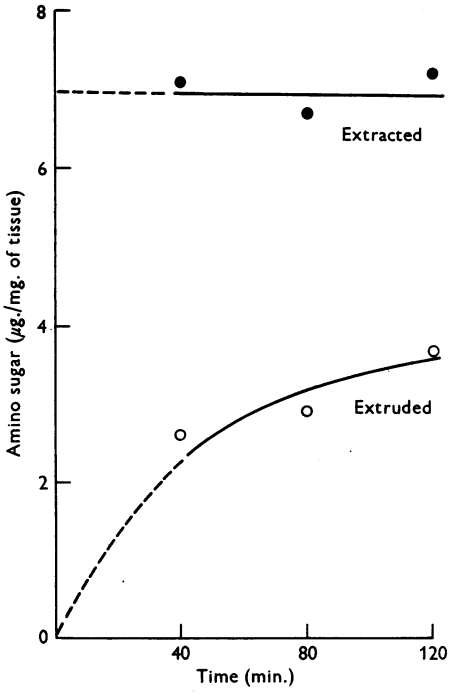

Fig. 3. Total amino sugar derivatives in the extruded (○) (i.e. soluble) and extracted (●) mucin fractions, formed during incubation of guinea-pig colon. Conditions of incubation and hydrolysis are given in Methods.

colon is one involving the formation of covalent ester linkages, rather than mere ionic exchange: thus [^{35}S]mucin does not lose [^{35}S]sulphate on dialysis or electrophoresis, nor is [^{35}S]sulphate fixed by mucin during incubation without tissue (Table 2). Furthermore, the incorporation process appears to be an enzymic one, requiring energy, since it is suppressed by heating or freezing the tissue (Table 2), by anaerobic conditions (Table 2), or by addition of inhibitors such as fluoroacetate (Table 3). The fact that incorporation follows a definite time course (Fig. 2) supports this view. Finally, it has been shown that the isotope is indeed present as bound [^{35}S]sulphate and that amino acids containing sulphur do not become labelled during incorporation (Fig. 1); this is in agreement with the findings of Kent, Whitehouse, Jennings & Florey (1956), that injected [^{35}S]-sulphate does not give rise to [^{35}S]amino acids in duodenal mucin obtained *in vivo*.

Such results as these have been obtained by workers studying incorporation of [^{35}S]sulphate by slices of cartilaginous tissue (Bostrom & Mansson, 1953; Kodicek & Loewi, 1955), and one may therefore conclude that certain aspects of the biosynthesis of intestinal mucin and chondroitin sulphate (formed in cartilage) are similar.

The present techniques enable closer study of the incorporation of [^{35}S]sulphate than hitherto; the overall process may be divided into three stages: (i) penetration of tissue by [^{35}S]sulphate; (ii) synthesis of [^{35}S]mucin; (iii) extrusion of [^{35}S]mucin. Penetration of tissue by [^{35}S]sulphate (stage i) is a rapid process, exchange being complete within 30 min. or less; this stage is stimulated by glucose (Table 3). Synthesis of [^{35}S]mucin (stage ii) and its extrusion (stage iii) have been followed over various time intervals during incubation (Figs. 2 and 3). It appears that extrusion is a faster process than synthesis, though the evidence is not conclusive. Extrusion is unaffected by glucose (Table 4), whereas synthesis is stimulated by glucose, glucosamine and pyruvate (Table 3).

Overall stimulation by glucose has been reported by several authors studying incorporation of [^{35}S]sulphate in cartilaginous tissue (Bostrom & Jorpes, 1954; Boyd & Neumann, 1954; Loewi & Kent, 1957). The extent to which synthesis of [^{35}S]mucin (stage ii) is so increased may be calculated in terms of [^{35}S]sulphate incorporated and compared with the amount of [^{35}S]sulphate which has penetrated the tissue (stage i). An increase of $1{\cdot}62 \times 10^{-2}$ μmole/mg. of dry tissue was found for incorporated sulphate in the presence of glucose, whereas the increase of free sulphate within the tissue was only $0{\cdot}085 \times 10^{-2}$ μmole/mg. of dry tissue. Stimulation of production of [^{35}S]mucin can therefore be only partly explained in terms of increased absorption of [^{35}S]sulphate by the tissue. It may be mentioned that the total amount of [^{35}S]sulphate incorporated in the presence of glucose is of the same order as that of free [^{35}S]sulphate in the tissue, indicating an appreciable turnover of the tissue-sulphate pool.

Stimulation of uptake of [^{35}S]sulphate by glucose is accompanied by an increase in Q_{O_2} (Table 3); Boyd & Neumann (1954), on the other hand, working with rat cartilage, reported a suppression of Q_{O_2}, whereas Loewi & Kent (1957) could detect no change in the respiration of guinea-pig granulation tissue in the presence of glucose. The effect of pyruvate (Table 3) is similar to that of glucose, causing stimulation of uptake of ^{35}S and Q_{O_2}; this suggests that the action of pyruvate, as of glucose, is concerned with supplementing the amount of energy available for synthetic processes (cf. Loewi & Kent, 1957). Stimulation of incorporation of ^{35}S by pyruvate, however, is not as marked as that by glucose. Glucose may therefore be affecting formation of [^{35}S]mucin by an additional mechanism, such as the formation of one of the precursors of [^{35}S]mucin; since galactose and glucosamine, both of which are constituents of intestinal mucin (Satoh, 1949; Werner, 1953; Whitehouse,

1955), are known to be formed from glucose under certain conditions (Leloir, 1955), this effect seems a likely one. It is supported by the observation that glucosamine itself is able to stimulate incorporation of [^{35}S]sulphate without altering Q_{O_2} (Table 3). This result is in accord with the finding of Bostrom, Roden & Vestermark (1955) and of Clark & Umbreit (1954), that glutamine is a powerful stimulant of incorporation of [^{35}S]sulphate by cartilage slices, since glutamine has been shown to be a possible amino-group donor in the glucose–glucosamine transformation (Pogell, 1956). We did not observe a glutamine effect (cf. Loewi & Kent, 1957); this discrepancy may be due to insufficient amounts of amino-group acceptors (such as glucose) within the tissue, or perhaps to a high level of glutamine in the tissue.

To summarize, the effects of glucose in stimulating synthesis of [^{35}S]mucin are: (*a*) increased penetration of tissue by sulphate; (*b*) additional energy is available for synthetic processes (cf. the action of pyruvate); (*c*) precursors of [^{35}S]mucin are formed and incorporated (cf. the action of glucosamine).

We think that the evidence which has been presented is sufficient to show that isolated segments of guinea-pig colon provide a useful system for the study of the biosynthesis of intestinal mucins. Subsequent investigations will be concerned with attempts to prepare active homogenates from this tissue and to isolate sulphate-containing intermediates.

SUMMARY

1. The mechanism of incorporation of [^{35}S]-sulphate by guinea-pig colon *in vitro* has been studied and found to be an enzymic process requiring energy.

2. Incorporated ^{35}S has been shown to be present as ester [^{35}S]sulphate in colonic mucin.

3. The effect of added substrates on respiration, penetration by [^{35}S]sulphate, incorporation of [^{35}S]-sulphate and total amino sugar content has been measured.

4. Sodium fluoroacetate, 2:4-dinitrophenol, phenol and salicylic acid inhibit respiration and incorporation of [^{35}S]sulphate.

5. Glucose stimulates respiration, penetration by [^{35}S]sulphate and incorporation of [^{35}S]sulphate; sodium pyruvate stimulates respiration and incorporation of [^{35}S]sulphate; glucosamine stimulates incorporation of [^{35}S]sulphate.

6. It has not been possible to decide whether the incorporation of [^{35}S]sulphate represents a net increase of mucin, or whether it is accompanied by an equivalent breakdown of preformed material.

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