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Biosynthesis of Intestinal Mucins

EFFECT OF PUROMYCIN ON MUCOPROTEIN BIOSYNTHESIS BY SHEEP COLONIC MUCOSAL TISSUE

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1. Surviving sheep colonic mucosal tissue incorporated L-[U-^{14}C]threonine when incubated in Krebs medium III at 37° in an atmosphere of oxygen, into a well-characterized mucoprotein fraction, isolated by papain digestion of the incubated scrapings. 2. Acidic hydrolysis and chromatography of the labelled mucoprotein showed that threonine was the only constituent to become labelled. In the presence of puromycin the incorporation of L-[U-^{14}C]threonine was considerably diminished (6·7 and 18·5% of control in duplicate experiments). Furthermore, puromycin also decreased incorporation of radioactivity from D-[U-^{14}C]-glucose (48·0 and 31·6% of control) and $^{35}SO_4^{2-}$ (21·2 and 23·6% of control) into the mucoprotein fraction. 3. In a puromycin-inhibited system, with D-[U-^{14}C]-glucose, where the overall specific radioactivity of the mucoprotein was 48% of control, the labelling of the individual monosaccharide constituents (as % of control) was: *N*-acetylneuraminic acid, 44%; *N*-glycollylneuraminic acid, 61%; hexosamines, 46%; fucose, 68%; galactose, 34%.

Previous work (Draper & Kent, 1963) has shown that sheep colonic scrapings are metabolically active *in vitro* and that in the presence of suitable biochemical energy-rich metabolites the cells incorporate labelled substrates into a major mucoprotein fraction. This fraction, isolated by papain digestion and preparative ultracentrifugation (Kent & Marsden, 1963), appears to have a structure consistent with that of other epithelial mucoproteins in that it consists of a central protein backbone carrying carbohydrate attachments (Kent, Ackers & Marsden, 1967). The present investigation has been concerned with the effects of puromycin on the biosynthesis of this mucoprotein and, in particular, on the rates of incorporation of threonine into the peptide moiety and of glucose into the monosaccharide of the carbohydrate portion of the molecule. A preliminary report of this work has been published (Allen & Kent, 1966*a*).

METHODS AND MATERIALS

The isolation of the colonic scrapings, the measurement of the Q_{O_2} and the isolation of the mucoprotein fraction were carried out as described by Draper & Kent (1963).

Radioactive incorporation experiments. Incubations were performed in stoppered flasks in an atmosphere of O_2 at 37° for 2½hr. Scrapings (2·5ml., corresponding to approx. 40–100mg. dry wt. of tissue/ml.) were added to modified Krebs medium III (Krebs, 1950) in a total volume of 10·1ml. In certain experiments the following radioactive compounds were added (in 0·1ml. of aqueous solution, the appropriate amounts being indicated in the respective Tables of results): D-[2-^{14}C]glucose (sp. radioactivity 28·4–31·3 μc/μmole), D-[U-^{14}C]glucose (sp. radioactivity 123–131 μc/μmole), L-[U-^{14}C]threonine (sp. radioactivity 25·6 μc/μmole) and carrier-free $Na_2{}^{35}SO_4$. All labelled substrates were obtained from The Radiochemical Centre, Amersham, Bucks. In some experiments the following additions were also made: 0·01% (w/v) polybactrin (obtained from Calmic Ltd., Crewe, Cheshire), or 0·01% (w/v) puromycin hydrochloride (obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A). Incubations were terminated by the addition of ethanol (3vol.). Zero-time incubations were performed in the same manner except that ethanol (3vol.) was added to the incubation medium before the addition of scrapings. With each radioactive incubation experiment, separate manometric determinations of the Q_{O_2} value were performed on a specimen of identical tissue (Draper & Kent, 1963).

Analysis of mucoprotein constituents. (*a*) Sialic acids. Sialic acid residues were cleaved from the mucoprotein by hydrolysis for 40min. with 0·1N-H_2SO_4 at 80° (Gottschalk, 1960). After cooling, the hydrolysis mixture was made neutral to methyl orange by addition of saturated $Ba(OH)_2$ and mixed with ethanol (3vol.). After 1hr. at 4°, the precipitated mucin and $BaSO_4$ were centrifuged and the supernatant was shaken with Dowex 50 (H^+ form) resin (1vol. of resin:5vol. of supernatant). The cation-free solution was decanted, and the resin was washed once with water (5vol.). The combined supernatant and washings were concentrated to dryness at 30mm. and

30°. The dried residue containing the sialic acids was dissolved in water (200 μl.) and analysed chromatographically on paper.

(*b*) Neutral sugars. Galactose and fucose were released from the mucoprotein by hydrolysis in 0·5 N-H_2SO_4 for 3 hr. at 105° (Mah, 1961). The resulting hydrolysate was passed down a column (1 cm. × 6 cm. for each millilitre of hydrolysate) of Amberlite Monobed 3 to remove mineral and sugar acids. The column eluate (about 100 ml.) was evaporated to dryness at 30 mm. and 40° and redissolved in water (200 μl.) for paper-chromatographic analysis.

(*c*) Amino sugars and amino acids. The mucoprotein fraction was hydrolysed in 2 N-HCl at 105° for 16 hr. (Blix, 1948). The resulting humin was centrifuged and the hydrolysate was evaporated to dryness *in vacuo* at room temperature over P_2O_5 and NaOH. This procedure was particularly suitable for the examination of the amino sugar constituents. Hydrolysis of the mucoprotein for amino acids was accomplished by treatment with 6 N-HCl at 105° for 24 hr. (Eastoe & Courts, 1963). The sample was prepared for chromatography by evaporation to dryness *in vacuo*.

In these and preceding hydrolyses, about 5·0 mg. of mucoprotein was employed/ml. of acid.

Paper-chromatographic systems. All paper-chromatographic analyses were carried out on Whatman no. 1 paper and, except where otherwise stated, by downward development. The following solvent systems were employed.

(*a*) For sialic acids. For all separations of these compounds the paper was first washed with 2 N-acetic acid for 24 hr., dried and then washed with glass-distilled water for a further 24 hr. Solvent systems used were: butan-1-ol–acetic acid–water (4:1:5, by vol.), ethanol–aq. NH_3 (sp.gr. 0·88)–water (40:1:10, by vol.) and ethyl acetate–acetic acid–water (3:1:3, by vol.). The most successful separations of *N*-glycollyl- and *N*-acetyl-neuraminic acid were achieved in the last-named solvent system by triple development, i.e. development with solvent three times, for 24 hr. each time, the chromatogram being dried between developments. Sialic acids were detected with orcinol (0·5%) in trichloroacetic acid (15%, w/v) in water-saturated butan-1-ol (Gottschalk, 1960), or alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) or benzidine–periodate (Cifonelli & Smith, 1954).

(*b*) For neutral sugars and hexosamines. The following solvent systems were employed: ethyl acetate–pyridine–water (2:1:2, by vol.), ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) and butan-1-ol–ethanol–water (5:1:4, by vol.). Neutral sugars were detected with aniline hydrogen phthalate (Partridge, 1949), or alkaline $AgNO_3$ or benzidine–periodate, as above. Free hexosamines were detected with alkaline $AgNO_3$ or acetylacetone–*p*-dimethylaminobenzaldehyde spray (Partridge, 1948) or ninhydrin, 0·1% (w/v), in water-saturated butan-1-ol containing 1% acetic acid (Consden & Gordon, 1948).

(*c*) For amino acids. The following solvent systems were employed: ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.), butan-1-ol–butanone–water (2:2:1, by vol.), and phenol–water (10:1, w/v) (this solvent was used for ascending chromatographic separations). Amino acids were detected with ninhydrin as above. Hexosamines were also analysed by the method of Gardell (1958).

Analytical methods. (*a*) Sialic acids. Sialic acids were estimated quantitatively in unhydrolysed mucoprotein fractions by the resorcinol method (Svennerholm, 1957). Direct determination of the sialic acid content of mucoprotein fractions was also carried out by the thiobarbituric acid method (Aminoff, 1961) after hydrolysis of the mucoprotein in 0·1 N-H_2SO_4 at 80° for 1 hr. The colorimetric procedure was carried out on the acid hydrolysate without further neutralization. Both these methods were suitable for the estimation of sialic acids in eluted chromatographic components.

(*b*) Neutral sugars. Fucose and galactose in eluates after chromatographic separation were determined by the reducing-sugar method of Somogyi (1952). Fucose was also determined by the thioglycollic acid method (Gibbons, 1955).

(*c*) Hexosamines. These were determined by the method of Levvy & McAllan (1959).

(*d*) Phosphorus. This was estimated by the method of Chen, Toribara & Warner (1956).

Radioactive assays. These were performed as described by Draper & Kent (1963). Eluted radioactive components from chromatograms (1–2 ml.) were evaporated (under an infrared lamp) to dryness on lens tissue on a 2·2 cm. diam. planchet.

RESULTS

Respiration of tissue

Incubation of the mucosal scrapings in Krebs medium III (D-glucose omitted, but L-glutamine, 3·2 mM, added) at 37° showed that the tissue maintained its metabolic activity over the period of 2½ hr. in which incorporation of radioactivity was studied. The average Q_{O_2} for the first hour was −3·75 (μl. of oxygen/hr./mg. dry wt. of tissue) and subsequently there was a progressive decrease in the oxygen uptake over the next 2 hr., the average Q_{O_2} values for the second and third hours being −2·93 and −2·42 respectively. The initial oxygen uptake varied considerably in individual experiments, the Q_{O_2} values for the first hour being between −6·20 and −2·18, depending on the freshness of the preparation.

Bacterial contamination. In prolonged incubations (e.g. over 4 hr.) of the tissue in Krebs medium III, an exponential rise in the gas uptake was observed (Fig. 1). This increase started to be appreciable in the fourth or fifth hour of incubation and although never observed before the fourth hour, its onset could be delayed up to the seventh hour by more prolonged washings of the colons before they were scraped; it was probably due to bacterial growth. A control without tissue, but containing the complete incubation medium, showed no uptake of gas over a period of 24 hr. Polybactrin (an antibiotic dusting powder containing, in amounts/g.: neomycin sulphate, 500 mg.; polymixin B sulphate, 100 000 units; zinc bacitracin, 25 000 units) in concentrations of 0·1–0·001% abolished the latter exponential uptake of gas with no marked effect on the oxygen uptake over the

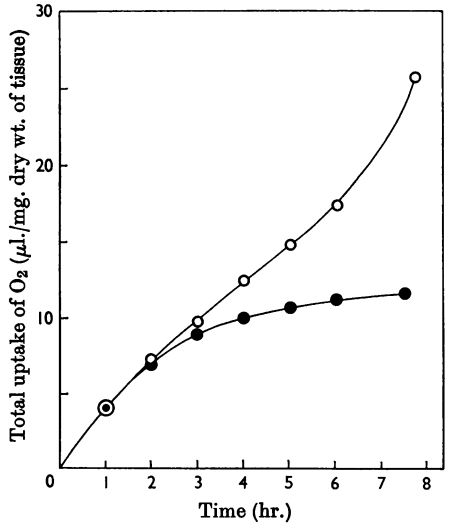

Fig. 1. Uptake of oxygen by sheep colonic mucosal tissue in the presence (●) and the absence (○) of puromycin (200 μg./ml.).

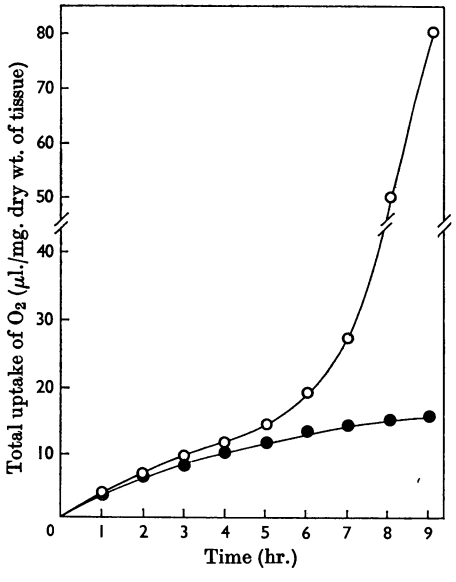

Fig. 2. Uptake of oxygen by sheep colonic mucosal tissue in the presence (●) and the absence (○) of polybactrin (0·01%).

first 2½hr. (three experiments) (Fig. 2). Polybactrin, moreover, did not markedly affect the incorporation of radioactivity from D-[2-^{14}C]-glucose or sodium [2-^{14}C]acetate into the mucoprotein fraction, and had only a slight effect on the incorporation of L-[U-^{14}C]threonine (Table 1). These results with polybactrin, together with the fact that an exponential rise in gas uptake never occurred before the fourth hour of incubation, were considered to make it unlikely that bacteria influenced the metabolic events of the tissue preparation recorded in the first 2½hr. of incubation. The structural studies (Marsden, 1964) have shown that the components of the papain-digested mucoprotein fraction are derived from larger macromolecular secretions of ovine epithelial (as opposed to bacterial) origin.

Radioactivity incorporation experiments

Utilization of D-[U-^{14}C]*glucose by mucosal tissue.* When D-[U-^{14}C]glucose (5 μc) was included in the incubation mixture and the mucoprotein fraction isolated as previously described (Draper & Kent, 1963), it was found that labelling was of the order of 6×10^3 and 10×10^3 counts/min./mg. dry wt. of tissue (Table 2). Acidic hydrolysis and chromatography, under appropriate conditions, led to the separation of the individual carbohydrate constituents. The constituents were eluted quantitatively and their radioactivity was measured (Table 3).

Electrophoresis on cellulose acetate strips of the radioactive mucoprotein fraction (in borate buffer, pH 9·3) gave three anionic components stainable with Alcian Blue. Draper & Kent (1963) and Kent & Marsden (1963) showed that the papain-digested mucoprotein fraction contained an electrophoretically slow-moving major component (fraction H), a slightly faster-moving component (fraction L) and a much faster-moving ribonucleic acid band. Only fraction H was found to bear sialic acid residues (J. C. Marsden & P. W. Kent, unpublished work), as well as some ester sulphate (3·5%), and to become labelled with radioactivity from D-[U-^{14}C]glucose. Fraction L appeared to be free from sialic acid residues and to owe its acidic character to the substantial amounts of ester sulphate. The absence of radioactive labelling in fraction L and in the ribonucleic acid means that the values quoted for the specific radioactivities in the present experiments must represent minimum values. The sialic acid content of the mucoprotein fraction provides an indication of the amount of fraction H present; the sialic acid content of fraction H by the thiobarbituric acid method was 6·8%, measured as *N*-acetylneuraminic acid (Kent & Marsden, 1963). Similarly, the presence of phosphorus in the mucoprotein fraction is indicative of the presence of nucleic acid (Draper & Kent, 1963).

Puromycin (100 μg./ml.) was included in simultaneous incubations with D-[U-^{14}C]glucose, samples

Table 1. *Effect of polybactrin* (0·01%) *on incorporation of radioactive substrates into a papain-digested mucoprotein fraction by sheep colonic mucosa*

Incubation medium (total volume 20·1 ml.) containing modified Krebs III medium (15·0 ml.; D-glucose omitted; L-glutamine, 3·2 mM, added), colonic tissue (5·0 ml.) and 5 μc of radioactive substrate (5 μc/0·1 ml.) was incubated for 2½ hr. at 37° under an atmosphere of O_2.

	Incubation medium		Mucoprotein fraction isolated	
Radioactive substrate	Polybactrin (%)	Uptake of O_2 (μl./mg. dry wt./2½ hr.)	Sp. radioactivity (counts/min./mg. ± S.D.)	Incorporation of added counts (%)
D-[2-^{14}C]Glucose	—	6·3	8945 ± 10·8	19·6
	0·01	—	8815 ± 11·8	20·9
L-[U-^{14}C]Threonine	—	3·6	305 ± 10·0	0·76
	0·01	—	244 ± 10·8	0·57
[2-^{14}C]Acetate	—	8·2	3955 ± 49·6	10·9
	0·01	—	3986 ± 35·1	9·1

Table 2. *Incorporation of* D-[U-^{14}C]*glucose into a papain-digested mucoprotein fraction by sheep colonic scrapings incubated with puromycin* (0·01%)

Incubation medium (total volume 10·1 ml.) containing modified Krebs III medium (D-glucose omitted; L-glutamine, 3·2 mM, added), tissue (2·5 ml.) and D-[U-^{14}C]glucose ($8{\cdot}11 \times 10^5$ counts/min. in 10·1 ml.) was incubated for 2½ hr. at 37°.

	Incubation medium	Mucoprotein fraction isolated			
Concn. of puromycin (%)	Uptake of O_2 (μl./mg. dry wt. of tissue/2½ hr.)	Sp. radioactivity (counts/min./mg. ± S.D.)	Total incorporation (% of counts added)	Phosphorus content (% dry wt.)	Sialic acid content† (% dry wt.)
Expt. 2P					
0	7·0	10240 ± 101	32·3	1·4	2·9
0·01	—	4920 ± 62	10·7	1·1	3·2
0 (zero time)	—	9·0 ± 6·1	—	1·8	3·8
Expt. 3P*					
0	11·7	6305 ± 54	17·0	0·8	4·4
0·01	—	1990 ± 25	5·4	0·7	4·3

* In Expt. 3P there was a 10 min. prior incubation with puromycin at 37° before D-[U-^{14}C]glucose was added.
† Sialic acid estimated as *N*-acetylneuraminic acid by the thiobarbituric acid method (Aminoff, 1961).

Table 3. *Incorporation of* [U-^{14}C]*glucose into monosaccharide constituents of a mucoprotein fraction from sheep colonic scrapings incubated with puromycin* (0·01%)

The hexosamines were estimated as glucosamine by the method of Levvy & McAllan (1959), and galactose by the method of Somogyi (1952) after chromatographic separation in ethyl acetate–pyridine–water. Sialic acids, chromatographically separated in ethyl acetate–acetic acid–water, were estimated by the method of Aminoff (1961) and fucose by the method of Gibbons (1955). For details see the Methods and Materials section.

	Expt. 2P			Expt. 3P		
	Control	Puromycin	Inhibition (% of control)	Control	Puromycin	Inhibition (% of control)
	(counts/min./mg.)			(counts/min./mg.)		
Mucin sample	10240 ± 10·1	4920 ± 62	48	6350 ± 54	1990 ± 25	32
Sialic acids	(10^{-3} × counts/min./μmole)			(10^{-3} × counts/min./μmole)		
N-Acetyl	2·14 ± 0·03	0·94 ± 0·08	44	2·22 ± 0·08	0·57 ± 0·7	26
N-Glycollyl	0·35 ± 0·04	0·21 ± 0·01	61	0·21 ± 0·02	0·10 ± 0·01	48
Hexosamines	7·52 ± 0·10	3·32 ± 0·07	46	3·60 ± 0·06	1·8 ± 0·05	50
Neutral sugars						
Fucose	2·64 ± 0·05	1·53 ± 0·06	58	4·26 ± 0·08	1·43 ± 0·06	33
Galactose	2·10 ± 0·04	0·72 ± 0·04	33	0·93 ± 0·03	0·20 ± 0·02	21

of the same tissue preparation as employed in the uninhibited controls (Tables 2 and 3) being used.

Incorporation of L-[U-^{14}C]*threonine into the mucoprotein fraction.* Incubation of mucosal tissue with L-[U-^{14}C]threonine (5 μC) under conditions identical with the preceding experiments led to labelling of the mucoprotein fraction (Table 4). Electrophoretic separation showed that only fraction H had acquired radioactive labelling from L-[U-^{14}C]-threonine. Acidic hydrolysis of the mucoprotein fraction and paper-chromatographic separation of constituents showed that threonine was the sole amino acid to become labelled. There was no labelling in the sialic acid, fucose, galactose, glucosamine and galactosamine residues. Paper chromatography with ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) of the hydrolysate (2N-hydrochloric acid for 16hr. at 105°) of the mucoprotein fraction showed labelling from L-[U-^{14}C]threonine in two peaks; one major peak (peak *A*), accounting for 90% of the total radioactivity added, ran with standard threonine (Fig. 3). The identity of this major radioactive peak, *A*, as threonine was confirmed by rechromatographing the eluted band in butan-1-ol–butanone–water (2:2:1, by vol.). The smaller radioactive peak, *B*, (R_{Thr} 0·51, 10% of total radioactivity added) did not resemble any of the common amino acids and, on further hydrolysis (2N-hydrochloric acid for 16hr. at 105°) and chromatographic analysis, it was found to contain aspartic acid, glycine or glutamic acid or both, serine, [^{14}C]threonine and small amounts of amino sugar. It would appear that this radioactive peak was an unhydrolysed glycoprotein fragment containing [^{14}C]threonine.

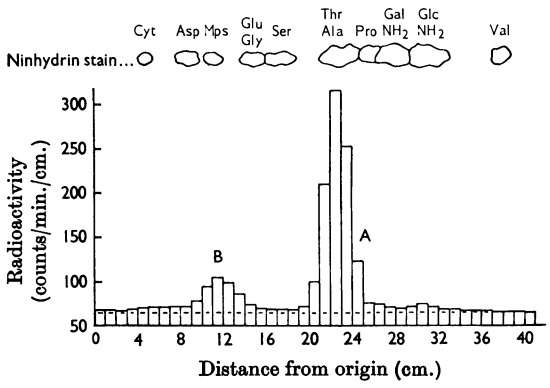

Fig. 3. Paper chromatography with ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.; development for 16hr.) of hydrolysate of mucin (2N-HCl for 16hr. at 105°) after incorporation of [U-^{14}C]threonine (sp. radioactivity 949 counts/min./mg.). Background counting rate (64 counts/min./cm.) is shown by the broken line. Cyt, cysteine; Mps, mucoproteins; $GalNH_2$, galactosamine; $GlcNH_2$, glucosamine.

The inclusion of puromycin (100 μg./ml.) in the incubation mixture caused a considerable decrease in the incorporation of the L-[U-^{14}C]threonine into the mucoprotein fraction by the mucosal tissue (Table 4).

Incorporation of $^{35}SO_4^{2-}$ *into the mucoprotein.* Incubations, under the conditions of the foregoing experiments, with $Na_2{}^{35}SO_4$ (25 μC), resulted in substantial labelling of mucoprotein fraction (Table 5). The label was entirely recoverable from the

Table 4. *Incorporation of* L-[U-^{14}C]*threonine into a papain-digested mucoprotein fraction isolated from sheep colonic scrapings incubated with puromycin* (0·01%)

Incubation medium (total volume 10·1 ml.) containing modified Krebs III medium (7·5 ml.) (D-glucose omitted; L-glutamine, 3·2 mM, added), tissue (2·5 ml.), and L-[U-^{14}C]threonine ($8{\cdot}11 \times 10^5$ counts/min. in 0·1 ml.) was incubated for 2½ hr. at 37°.

Incubation medium		Mucoprotein fraction isolated			
Concn. of puromycin (%)	Uptake of O_2 (μl./mg. dry wt. of tissue/2½ hr.)	Sp. radioactivity (counts/min./mg. ± S.D.)	Total incorporation (% of counts added)	Phosphorus content (% dry wt.)	Sialic acid content† (% dry wt.)
Expt. 1P*					
0	6·8	949 ± 20·6	3·25	1·8	4·51
0·01	—	64 ± 6·3	0·36	1·7	4·9
0 (zero time)	—	5·2 ± 5·2	—	1·3	5·7
Expt. 2P					
0	8·5	486 ± 15·5	0·98	1·0	4·7
0·01	—	90 ± 11·7	0·11	1·5	4·3
0 (zero time)	—	4·0 ± 4·0	—	1·1	4·7

* In Expt. 1P there was a 10 min. prior incubation with puromycin at 37° before L-[U-^{14}C]threonine was added.

† Sialic acid estimated as *N*-acetylneuraminic acid by the thiobarbituric acid method (Aminoff, 1961).

Table 5. *Incorporation of $^{35}SO_4^{2-}$ into a papain-digested mucoprotein fraction isolated from sheep colonic mucosal tissues incubated with puromycin* (0·01%)

Incubation medium (total volume 10·1 ml.) containing modified Krebs III medium (D-glucose omitted; L-glutamine, 3·2 mM, added), tissue (2·5 ml.), and $^{35}SO_4^{2-}$ ($40·5 \times 10^5$ counts/min. in 0·1 ml.) was incubated for 2½ hr. at 37°.

Incubation medium		Mucoprotein fraction isolated			
Concn. of puromycin	Uptake of O_2 (μl./mg. dry wt. of tissue/2½ hr.)	Sp. radioactivity (counts/min./mg. ± S.D.)	Total incorporation (% of counts added)	Phosphorus content (% dry wt.)	Sialic acid content† (% dry wt.)
Expt. 3P*					
0	11·7	2500 ± 41·0	1·12	1·3	4·7
0·01	—	526 ± 12·0	0·39	0·8	4·7
0·01 (zero time)	—	8 ± 3·5	—	1·4	5·3
Expt. 4P					
0	7·0	3240 ± 51·0	1·38	1·6	3·5
0·01	—	765 ± 25·0	0·21	1·0	4·2
0 (zero time)	—	3 ± 5·8	—	1·4	3·7

* In Expt. 3P there was a 10 min. prior incubation with puromycin at 37° before $^{35}SO_4^{2-}$ was added.
† Sialic acid estimated as *N*-acetylneuraminic acid by the thiobarbituric acid method (Aminoff, 1961).

mucoprotein fraction as inorganic $^{35}SO_4^{2-}$, in contrast with the non-labelled sulphur-containing amino acids. These results agree with those of Draper & Kent (1963), where it was shown that $^{35}SO_4^{2-}$ was incorporated into fraction H solely as ester sulphate. The presence of puromycin (100 μg./ml.) caused a marked decrease in the incorporation of $^{35}SO_4^{2-}$ into the mucoprotein fraction (Table 5).

Experiments with puromycin. Puromycin (200 μg./ml.) did not noticeably affect the oxygen uptake of the tissue over the period of the radioactive incubations (2½ hr.). However, puromycin appeared to act as a bacteriostatic agent, abolishing the exponential rise in gas uptake beginning in the fifth hour of incubation in the control.

Electrophoresis in borate buffer, pH 9·3, of the mucoprotein fraction isolated from incubations of puromycin with any one of the three radioactive substrates used above (D-[U-^{14}C]glucose, L-[U-^{14}C]-threonine or $^{35}SO_4^{2-}$) showed that the components and in particular fraction H were of mobility identical with those of the control sample. The samples from the puromoycin incubations contained only one radioactive component and this had the same mobility as fraction H.

DISCUSSION

The colonic epithelium of mammals is prolific in mucus production (Florey, 1962) and presents a suitable system for the investigation of mucin biosynthesis (Kent, 1962). The principal mucoprotein (fraction H) of the fraction remaining after papain digestion of sheep colonic scrapings is derived from a mucoprotein that is the major component of the untreated aqueous mucus extract of the tissue (Marsden, 1964). Fraction H has been isolated, free of the other constituents of the papain-digested mucoprotein fraction, by preparative ultracentrifugation (Kent & Marsden, 1963) and shown to have a structure consistent with that of an inner peptide core to which an arrangement of relatively short oligosaccharide side-branches terminating in sialic acids (*N*-acetyl- and *N*-glycollyl-neuraminic acid) and fucose are attached. The major penultimate sugar is galactose, which is attached to an inner core of *N*-acetylglucosamine and *N*-acetylgalactosamine residues, the hexosamine residues being involved in the carbohydrate–peptide attachments and also considered to be the site of attachment of the ester sulphate substituents (Kent *et al.* 1967). In principle therefore, fraction H resembles structurally other epithelial mucoproteins such as ovine and bovine submaxillary mucins (e.g. Gottschalk, 1963; Hashimoto, Tsuiki, Nisizawa & Pigman, 1963), the blood-group substances (e.g. Morgan & Watkins, 1959; Watkins, 1966) and cervical mucin (Gibbons & Roberts, 1963).

In labelling experiments, the tissue incorporated L-[U-^{14}C]threonine (3·25 and 0·98% of the total counts added) into fraction H solely as threonine. L-Threonine was chosen as the amino acid for investigation of the biosynthesis of the protein moiety of mucin for two reasons. First, as it is an essential amino acid (Meister, 1965) its amount in the tissue would be expected to be unaffected by the amount of other metabolites in the tissue. Secondly, threonine (5·5%, w/w) is the most abundant amino acid in fraction H (Kent, Draper,

Table 6. *Summary of effects of puromycin (0·01%) on incorporation of radioactive precursors of a papain-digested mucoprotein fraction by sheep colonic mucosal tissue*

All results are expressed as % of control.

	L-[U-^{14}C]Threonine		D-[U-^{14}C]Glucose			
Expt.	Sp. radio-activity	Total incorporation	Sp. radio-activity	Total incorporation	Sp. radio-activity	Total $^{35}SO_4^{2-}$ incorporation
1P*	6·7	10·9	—	—	—	—
2P	18·5	11·6	48·0	33·2	—	—
3P*	—	—	31·6	31·8	21·1	34·6
4P	—	—	—	—	23·6	15·2

* In Expts. 1P and 3P there was 10 min. prior incubation with puromycin at 37° before the radioactive substrate was added.

Marsden & Allen, 1964) and may occupy the key position in the linkage of the carbohydrate groups to the peptide chain.

Incubation of tissue with $^{35}SO_4^{2-}$ produced labelled mucin (1·12 and 1·38% of the total added counts), the radioactivity being incorporated solely into the ester sulphate of fraction H.

The incorporation of D-[U-^{14}C]glucose into all the monosaccharide constituents, of L-[U-^{14}C]-threonine into the peptide portion, and of $^{35}SO_4^{2-}$ into the ester sulphate residues of the single metabolically-active fraction of mucin, fraction H, suggests that under the conditions of the incubations actual biosynthesis of this fraction is occurring rather than an exchange in the constituents of preformed fraction H. Other studies (Draper & Kent, 1963; Kent & Allen, 1968) showing the incorporation of D-[1-^{14}C]- and D-[2-^{14}C]—glucose, [1-^{14}C]- and [2-^{14}C]-acetate, [3-^{14}C]pyruvate and [3-^{14}C]hydroxypyruvate into the monosaccharide residues, and L-[U-^{14}C]serine and [U-^{14}C]glycine into the peptide moiety of fraction H, are consistent with the biosynthesis of this mucoprotein resulting under the conditions of the incubation. The present investigations with puromycin are concerned with the extent to which the biosynthesis of the full mucoprotein structure is contingent on the initial formation of the polypeptide backbone, addition of the carbohydrate residues (singly or as oligosaccharides) occurring subsequently.

Puromycin has been shown to have a specific inhibitory effect on the biosynthesis of proteins at the ribosomal level (Yarmolinsky & de la Haba, 1959). It is to be expected that puromycin might also inhibit biosynthesis of the peptide portion (and dependent aspects) of mucoproteins. Inhibition (93·5 and 81·5%, Table 6) of L-[U-^{14}C]threonine incorporation into mucin by puromycin is consistent with this view. Further, the inhibitory effect on the threonine incorporation is evidence for the view that the biosynthesis of the peptide portion of fraction H proceeds by a pathway similar to that found for the biosynthesis of other proteins in mammals (e.g. Campbell, 1965).

At the same time puromycin inhibited the incorporation of D-[U-^{14}C]glucose and $^{35}SO_4^{2-}$ into mucin, albeit to a lesser extent than that of threonine. D-[U-^{14}C]Glucose incorporation is the least affected (68·4 and 52·0% inhibition, Table 6) and $^{35}SO_4^{2-}$ somewhat more (78·9 and 76·4% inhibition, Table 6). Analysis of mucin from labelling experiments with D-[U-^{14}C]glucose in the presence of puromycin showed that the decrease in incorporation was reflected non-specifically in all the monosaccharide constituents (Table 3). This is in contrast with the action of salicylate on this tissue (Allen & Kent, 1966*b*), which, at partially inhibitory concentrations, inhibits extensively the utilization of D-[2-^{14}C]glucose in the formation of the amino sugar constituents whereas the formation of the neutral sugars is comparatively unaffected. It appears therefore that the action of puromycin is a general one as far as the incorporation of D-[U-^{14}C]glucose into the monosaccharides is concerned.

These results are complementary with those of other workers showing that intravenous puromycin inhibits the incorporation of [^{14}C]glucosamine into protein of rat liver (Molnar, Robinson & Winzler, 1964) and into the protein of Ehrlich ascites-tumour cells (Cook, Laico & Eylar, 1965). The present system has the advantage that the biosynthetic studies can be related to a mucoprotein of defined structure. Studies *in vitro* by other workers have also shown that puromycin has an inhibitory action on the biosynthesis of the carbohydrate fragment of mucopolysaccharides. de la Haba & Holzer (1965) have shown that puromycin inhibits the incorporation of [^{14}C]valine, D-[^{14}C]glucose, and $^{35}SO_4^{2-}$ into chondroitin sulphate by chick-embryo chondrocytes. Inhibition by puromycin of the incorporation of radioactivity from D-[^{14}C]-

glucosamine and L-[^{14}C]threonine into bovine submaxillary mucin by tissue slices has been demonstrated by Lawford & Schachter (1965).

The inhibitory effect of puromycin on the biosynthesis of the carbohydrate constituents of mucoprotein suggests that the formation of the protein backbone precedes, and is essential for, the subsequent linking of the carbohydrate residues. However, other possibilities exist; for example, it is possible that puromycin may inhibit enzymes involved in cell metabolism other than those of ribosomal protein synthesis. Moreover, if the turnover of the enzymes of the cell is rapid, puromycin may decrease the amount of enzyme protein necessary for optimum metabolic conditions. Evidence from other workers does not support these possibilities. The work of Kornfeld, Kornfeld, Neufeld & O'Brien (1964) and Molnar *et al.* (1964), on the distribution of radioactivity from intravenously injected [^{14}C]glucosamine in the soluble monosaccharide precursors of rat-liver glycoproteins, is consistent with a single biochemical effect of puromycin, namely that on protein biosynthesis. Moreover puromycin does not interfere with the release of radioactive leucine by rat-liver slices (de la Haba & Holzer, 1965). The absence of inhibitory effects of puromycin on the particulate polymerases catalysing the incorporation of sugar nucleotides into glycoprotein precursors (e.g. O'Brien, Canady & Neufeld, 1965; Sarcione & Carmody, 1966) is also in keeping with a specific action of this inhibitor. Finally, in the work reported in this paper no inhibitory effect of puromycin on the oxygen uptake by the tissue over the 2½hr. incubation period was observed.

There is as yet no evidence of any structurally modified mucoprotein being formed in the presence of puromycin. Electrophoresis in borate buffer, pH 9·3, showed no discernible difference between mucin from control and puromycin experiments with all radioactively labelled substrates, there being only one radioactive band moving with fraction H. The greater inhibition by puromycin of the threonine incorporation compared with glucose suggests that a pool of peptide precursor exists *in vivo*. Attempts to decrease this pool of precursor by preincubation for 10min. with puromycin before the addition of radioactive tracer caused a greater decrease in the specific radioactivity, but this was not so with the decrease of the percentage incorporation of total added counts (Table 6), and further experiments will be necessary to establish this point.

In short the above evidence is consistent with the suggestions that puromycin acts at the site of ribosomal protein biosynthesis in sheep colonic mucosal mucin formation and that the biosynthesis of the carbohydrate portion is strongly dependent on the prior formation of peptide. The finding that puromycin affects the sulphate and carbohydrate utilization to a somewhat similar degree suggests that the sulphation step, i.e. transference to the carbohydrate acceptors, takes place during the biochemical assembly of the mucoprotein rather than later with the fully formed macromolecule.

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