other moving faster (mobility 0·52). This suggested incomplete cleavage at the second stage, resulting in the production of CPOψ2 together with CPOψ3 at the third stage. Cleavage appeared to be complete at all subsequent stages, although two peptides were always obtained, owing to the incomplete cleavage of the second stage. The DNS-CPOψ4 (mobility 0·56) and DNS-CPOψ5 (mobility 0·78) were obtained in reasonable yield. The DNS-CPOψ6 was identified as a very weak band (mobility 0·94), moving in the tail of the dimethylaminonaphthalenesulphonic acid band. Subsequent stages were unsuccessful, since labelling was inhibited by the presence of an accumulation of oxidized pyridine products.

After hydrolysis of the bands, each gave rise to a single fluorescent spot at pH 4·40, corresponding to the *N*-terminus of the peptide. In order, the residues identified were DNS-Gly, DNS-Pro, DNS-Ileu, DNS-Pro, DNS-MetSO_2 and DNS-Pro. DNS-CPOψ6 did not give an identifiable product. Thus the *N*-terminal sequence was established as Gly-Pro-Ileu-Pro-Met-Pro-, in complete agreement with the conclusions reached by the larger-scale methods used by Ambler (1963).

Generally it is not necessary to purify the DNS-peptides before hydrolysis, and this may be undesirable when only very small amounts (1 μmole) are available. Here the large number of proline residues present made it desirable to obtain additional information, such as extent of cleavage and mobilities of the DNS-peptides. These mobilities were inversely proportional ($\pm 5\%$) to the molecular weights of the DNS-peptides, so that useful confirmation of the proposed structures was obtained in this way.

SUMMARY

1. A micro-technique for stepwise sequential analysis of small quantities of peptide is outlined.

2. By applying this method to 0·02 μmole of a chymotryptic peptide from *Pseudomonas* cytochrome *c*-551, the *N*-terminal sequence

Gly-Pro-Ileu-Pro-Met-Pro-

was determined.

W. R. Gray is indebted to the Medical Research Council for a scholarship held during this work.

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The Sensitivity of the Neuraminosidic Linkage in Mucosubstances towards Acid and towards Neuraminidase

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Neuraminic acid may readily be split from substances containing it either with 0·1N acid at 80° or with the enzyme neuraminidase. The rate and extent of hydrolysis may conveniently be followed by means of the thiobarbituric acid reaction (Warren, 1959; Aminoff, 1961), which estimates only the free acid. Generally, acidic hydrolysis is rapid; after about 1 hr. it is maximal and has usually been assumed to be quantitative. Likewise the liberation of neuraminic acid by neuraminidase is often found to go essentially to completion, though in some substances, particularly those from epithelial mucus, there is some residual neuraminic acid relatively resistant to this enzyme (Warren & Spicer, 1960).

This investigation originated with the observation that bovine cervical mucopolysaccharide behaved anomalously on hydrolysis in 0·1N acid at 80°; the shape of the hydrolysis curve (amount of neuraminic acid estimated by thiobarbituric acid against time) was not that predicted for the second member of a sequential series of first-order irreversible reactions; the time of maximal hydrolysis was 2·5 hr., and the maximum amount released, as estimated by thiobarbituric acid with *N*-acetylneuraminic acid as standard, was only 65% of the amount estimated by the direct Ehrlich's reaction (Werner & Odin, 1952). Furthermore, only an apparent 38% of the total neuraminic acid was removed at all readily with neuraminidase.

Some other highly purified preparations have recently been reported to give low results after hydrolysis for 1 hr. in the standard thiobarbituric acid analysis, as compared with the Ehrlich's or resorcinol (Svennerholm, 1957) estimations: for example, colominic acid (Aminoff, 1961), ganglioside (Balakrishnan & McIlwain, 1961) and bovine submaxillary mucoprotein (Hashimoto, Tsuiki, Nisizawa & Pigman, 1963). This last-named substance has also been found to yield less than half of its neuraminic acid on hydrolysis with neuraminidase (Faillard, 1957). Faillard (1959) has also shown that the greater part, though apparently not all, of the neuraminidase-resistant neuraminic acid is *O*-acetylated; a sample of bovine submaxillary mucoprotein that has been de-*O*-acetylated by mild alkaline treatment yields a little over 90% of its total neuraminic acid under the influence of neuraminidase. This observation has been confirmed, and anomalies in the hydrolysis curve of this substance may be largely removed by alkaline treatment. Preliminary experiments indicated that the neuraminidase-resistant neuraminic acid in bovine cervical mucopolysaccharide was not the same as that in bovine submaxillary mucoprotein, since the 0·1N acid-hydrolysis curve was not detectably altered by treatment with alkali.

In an attempt to throw some light on the nature of the neuraminosidic linkages in bovine cervical mucopolysaccharide their hydrolysis in 0·1N acid at 80° and by neuraminidase was studied, and compared with the behaviour of the neuraminosidic linkages in a number of other substances under the same conditions. In the course of this work the decomposition of neuraminic acid in acid was also studied and found to be complex. A preliminary report on this point has already been made (Gibbons, 1962*a*). The results of this study are reported and their structural implications discussed.

EXPERIMENTAL

Materials

Bovine cervical, human cervical and human bronchial mucopolysaccharides. These materials were obtained by the general procedure described by Gibbons (1959). The starting materials were: (*a*) pooled bovine cervical mucus plugs from pregnant animals; (*b*) similarly pooled material collected from animals at or near oestrus; (*c*) pooled human midcycle cervical mucus; (*d*) a 24 hr. collection of sputum from a patient with chronic bronchitis. Rather prolonged periods of extraction with $CaCl_2$–ethanol reagent, up to 14 days, have been used, but with the two human materials small amounts of material, largely cellular debris, remained undissolved and were discarded. With the human cervical material, the 40–50% and 50–60% ethanol precipitates were combined to give sufficient material for study. Fractionation from $CaCl_2$ solution with ethanol was carried out three times in each case. Both bovine materials are single components electrophoretically at pH 5·75 (lithium acetate *I* 0·1) and in the ultracentrifuge (pH 7·3, 0·01M-phosphate, 0·2M-NaCl). The other two samples have not been examined by these techniques.

Bovine submaxillary mucoprotein. This material was prepared according to Heimer & Meyer (1956). It behaves as a single component electrophoretically at pH 5·75 (lithium acetate, *I* 0·1) and pH 8·5 (barbiturate–NaCl, *I* 0·1) and in the ultracentrifuge at pH 7·3 (0·01M-phosphate, 0·2M-NaCl). Its $S^0_{20,w}$ by extrapolation of $1/S$ against concentration is $5{\cdot}4 \times 10^{-13}$ sec., and the intrinsic viscosity is 2·05 dl./g. The diffusion coefficient is $1{\cdot}45 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 0·35% concentration.

Goat and pig submaxillary mucoprotein. The minced submaxillary glands were extracted by shaking mechanically three times with 5 vol. of 6M-urea at room temperature. The extract was diluted with an equal volume of water, and 1 ml. of aq. 10% (w/v) cetyltrimethylammonium chloride/100 ml. of extract was added. After some hours at 0–4° the precipitate was collected and extracted five successive times at room temperature with stirring with an appropriate volume of methanol. The combined methanol extracts were treated with a saturated solution of LiCl in ethanol until precipitation was complete. The precipitate was twice dissolved in 2M-LiCl and precipitated with 10 vol. of ethanol. The precipitate was then dissolved in water and shaken twice for 2–3 hr. with one-half of its volume of pentanol–chloroform (1:3, v/v). The aqueous phase was dialysed and freeze-dried. Both preparations behaved as single components on electrophoresis (pH 5·75, lithium acetate, *I* 0·1).

Neuramin-lactose. This preparation was obtained from 1 l. of defatted bovine colostrum essentially as described by Kuhn & Brossmer (1956). It was not crystalline, but when analysed for neuraminic acid (direct Ehrlich's reaction), lactose (anthrone) and nitrogen (Kjeldahl) it gave 95–98% of the theoretical values. It was chromatographically homogeneous (ethyl acetate–acetic acid–water, 3:1:3) but paper electrophoresis showed evidence of a second component, which was not free neuraminic acid.

Neuraminidase. A small amount of material was prepared from *Vibrio cholerae* culture filtrates as described by Schramm & Mohr (1959). After final dialysis 1 vol. of 0·5M-NaCN neutralized with dilute acetic acid was added to 99 vol. of the active solution, which was stored at −10°. It contained approximately 12 μg. of protein N/ml. This preparation was used to digest bulk samples of the bovine cervical and submaxillary materials for isolation and further study. The amount of added enzyme was insignificant compared with that of the substrate, which allowed the isolation of the treated substances by dialysis. Most of the other experimental work reported with this enzyme was carried out with a commercial enzyme solution (Burroughs Wellcome and Co.). The commercial enzyme contains extraneous inactive protein but behaves towards the two bovine materials, goat submaxillary mucoprotein and neuramin-lactose, in the same manner as the purified enzyme. It is devoid of detectable aldolase.

Calf stroma. Washed calf erythrocytes were lysed osmotically and their stroma washed until supernatants were colourless. The preparation was used as a fine suspension.

Methods

Analytical methods. Nitrogen, hexosamine, hexose and fucose were estimated as described by Gibbons (1959). Free neuraminic acid was estimated by the thiobarbituric

acid method of Warren (1959) or Aminoff (1961). These methods are very similar and were used interchangeably; that of Aminoff is somewhat more sensitive. The direct Ehrlich's reaction described by Werner & Odin (1952) and the direct resorcinol reaction of Svennerholm (1957) were also used to estimate total neuraminic acid. Other methods used for estimating free neuraminic acid were the column separation procedure of Svennerholm (1958) and the alkaline Ehrlich's procedure of Aminoff (1961). Total glycolloyl residue was estimated as described by Gibbons (1962*b*), and *O*-acetyl (ester groups) by the hydroxamic acid method of McComb & McCreedy (1957) with crystalline octa-*O*-acetylmaltose as standard. Mucosubstances sometimes give a troublesome turbidity in this procedure that makes the method unreliable at low ($< 0{\cdot}1\%$) contents of *O*-acetyl.

N-Acetylhexosamine reaction. This was carried out as described by Aminoff, Morgan & Watkins (1952). Water (1 ml.) was added to each tube (including standards), before reading, to eliminate cloudiness.

Physical examinations. The two bovine substances were characterized physically as described by Gibbons & Glover (1959). Free electrophoresis was carried out by the usual technique with a Tiselius apparatus (Perkin–Elmer Ltd.).

Paper chromatography. This was carried out by using the usual descending technique, with ethyl acetate–acetic acid–water (3:1:3, by vol.) or butan-1-ol–propan-1-ol–0·1N-HCl (1:2:1, by vol.) as solvents. The latter is preferable. The dry chromatograms after irrigation were dipped in orcinol–trichloroacetic acid or dimethylaminobenzaldehyde reagents to locate the neuraminic acid (Gottschalk, 1960).

Acid-breakdown products of neuraminic acid. Neuraminic acid (10 mg.) in 2·5 ml. of 0·1N-H_2SO_4 was heated at 80° for 6 hr. The very dark solution was passed down a column (1 cm. diam. × 10 cm.) of De-Acidite FF (The Permutit Co. Ltd.) in its acetate form. The column was washed with water and the first 20 ml. of the eluate was collected in a graduated vessel. Of this, 14 ml. was withdrawn for direct Ehrlich's, resorcinol and thiobarbituric acid assay; the remainder was adjusted to pH 7·5 with 0·5M-tris. This solution was placed in an incubator at 37° and samples were periodically withdrawn for thiobarbituric acid analysis.

Hydrolysis curves. Solutions (or suspensions) of the material to be studied in 5–10 ml. of 0·1N-H_2SO_4 were prepared such that the maximum concentration of neuraminic acid released was appropriate to the analytical procedure to be used (40–80 μg./ml. for the thiobarbituric acid method). Hydrolysis was carried out in a glass-stoppered tube in a water bath thermostatically regulated. Samples were removed at appropriate times and stored at −10° until analysed. To determine the extent of possible sources of systematic error in this procedure, various modifications were assayed. Thus separate experiments in which (*a*) a buffer (0·5M, pH 1·25) was used in place of 0·1N-H_2SO_4, (*b*) the solution and hydrolytic agent were pre-warmed before mixing and (*c*) the samples were cooled to room temperature before being pipetted for analysis, revealed that changes in pH during hydrolysis, the finite time taken to reach thermostat temperature at the commencement of the experiment and the pipetting of warm solutions did not give rise to significant error. It was also found, by carrying out the hydrolysis in separate sealed ampoules, that evaporation in the test solution when the stopper is removed for sampling does not contribute significantly to the error, provided that by the time all samples have been withdrawn an adequate volume (> 3 ml.) still remains in the tube. Having regard to the analytical error of the thiobarbituric acid method ($\pm 1{\cdot}9\%$ S.D.; see Table 1) none of the refinements described appears to be worth while, and they were not used.

Detection of aldolase in commercial neuraminidase. A portion (1 ml.) of 1% (w/v) neuraminic acid in acetate buffer, pH 5·75, *I* 0·1, was treated with (*a*) 2 ml. of commercial neuraminidase and (*b*) 2 ml. of the same solution that had been heated at 100° for 10 min. Both solutions were incubated overnight and assayed for neuraminic acid and *N*-acetylhexosamine.

Alkaline treatment of bovine mucosubstances. The materials (50 mg.) dissolved in 50 ml. of 0·05M-Na_2CO_3 were heated at 100° for 20 min. After cooling they were dialysed for 6 days at 0–4° against 1 l. of distilled water, which was changed daily. The indiffusible material was recovered by freeze-drying.

Action of neuraminidase. (1) Bovine cervical mucopolysaccharide (300 mg.) or bovine submaxillary mucoprotein (250 mg.), dissolved in 20 ml. of acetate buffer, pH 5·7, *I* 0·02, was incubated with 2 ml. of purified enzyme solution and one or two drops of toluene at 37° for 22 hr. Samples were withdrawn and analysed for neuraminic acid (thiobarbituric acid method) after 15 hr. and 22 hr. and were found to differ by less than 2%; 47 and 44% respectively of the total neuraminic acid (corrected for glycolloyl content) had been liberated. The samples were then dialysed free of salt and neuraminic acid and dried.

(2) An amount of material to be examined containing 0·5–1 mg. of neuraminic acid dissolved in 2 ml. of water was added to 1 ml. of acetate buffer, pH 5·7, *I* 0·1, and to it was added 2 ml. of commercial enzyme preparation and one drop of toluene. The materials were incubated at 37°. Samples (0·1 ml.) were withdrawn for analysis (thiobarbituric acid method) over a period of 46 hr. From the solution remaining, two samples (0·5 ml.) were withdrawn, one of which was placed in a tube containing 0·05 ml. of a 1% solution of goat submaxillary mucoprotein and the other in a tube containing 0·05 ml. of water. Both solutions were incubated for a further 4 hr. Water (1·5 ml.) was then added to each tube and two samples (0·2 ml.) were removed for analysis by the thiobarbituric acid method. From the solution remaining after withdrawal of the two samples (0·5 ml.), a further 2 ml. was withdrawn and dialysed for 6 days at 0–4° with daily changes of distilled water. At the end of this time, the contents of the dialysis tubes were washed quantitatively into 15 ml. graduated flasks, which were made up to the mark and from which two samples (5 ml.) were withdrawn for direct Ehrlich's assay.

Isolation of products of mild acid hydrolysis. Bovine cervical mucopolysaccharide (200 mg.) was dissolved in 20 ml. of 0·1N-H_2SO_4 and heated at 80° for 50 min. The cooled solution was titrated with saturated aqueous $Ba(OH)_2$, with stirring, to pH 7·5. The precipitate was removed and washed; the supernatant and washings were then dialysed at room temperature against eight changes of about 65 ml. of distilled water saturated with chloroform. The pooled diffusates were evaporated at low pressure to about 50 ml., when a sample (4 ml.) was removed for examination and the remainder was freeze-dried; yield 28 mg. A small column (0·7 cm. × 10 cm.) packed with a mixture of charcoal and kieselguhr (3:1) was well washed with water and the diffusate dissolved in about 1 ml. of

water was introduced on to the top of the column. The first fraction (F1, 10 mg.) was eluted with 20 ml. of water; a second fraction (F2, 10 mg.) was eluted with a similar amount of 10% (v/v) ethanol and a third fraction (3 mg.) with 50 ml. of 25% (v/v) ethanol. A further small amount was recovered by washing with 200 ml. of ethanol–phenol–water (4:1:5, by vol.) but was not isolated. Fractions were isolated by freeze-drying.

Reduction with sodium borohydride. Five solutions for test were prepared containing 4 mg. of the fractions F1 and F2 from mild acid hydrolysis described above, 2 mg. of neuraminic acid, 5 mg. of neuramin-lactose and 10 mg. of goat submaxillary mucoprotein respectively in 2 ml. of water. To these solutions 2·5 ml. amounts of a buffer containing 5·3 g. of Na_2CO_3 and 8·4 g. of $NaHCO_3$/l. were added. Two samples (2 ml.) were then withdrawn from each experimental sample and placed in centrifuge tubes graduated at 10 ml. The solutions in the first (control) set of five tubes were neutralized with H_2SO_4 and made up to 10 ml. To the second set of five tubes 40 mg. of solid $NaBH_4$ was added and the solutions were allowed to stand at room temperature with occasional inversion for 1 hr. The contents of the tubes were then acidified by addition of 1 ml. of N-H_2SO_4 and, after standing for a further 5 min., were neutralized (indicator paper) by dropwise addition of saturated $Ba(OH)_2$ to pH 7–8. The tube contents were then made up to 10 ml.; the precipitated $BaSO_4$ and borate were packed down by centrifuging and two samples (4 ml.) of the supernatants were removed for analysis by the direct Ehrlich's assay. Two portions (4 ml.) were similarly removed from the control tubes.

Calculations

The standard equation (see, for example, Frost & Pearson, 1961) for the second component of a series of sequential first-order irreversible reactions $A \rightarrow B \rightarrow C$ is:

$$q = \frac{K_1 a}{K_1 - K_2}(e^{-K_2 t} - e^{-K_1 t}) \quad (1)$$

where q is the amount of component B observed, t is time, K_1 and K_2 are the specific reaction rates for the first and second reactions respectively and a is the total amount of A available for reaction.

By differentiating and equating to zero the relation:

$$t_{max.} = \frac{2{\cdot}303 \log(K_1/K_2)}{(K_1 - K_2)} \quad (2)$$

and the further relation:

$$K_2 t_{max.} = 2{\cdot}303 \log\left(\frac{a}{q_{max.}}\right) \quad (3)$$

may be obtained. ($q_{max.}$, Maximum value of component B; $t_{max.}$, time at which that value is reached.)

The experimental curve is plotted and values $t_{max.}$ and $q_{max.}$ are extracted. The value K_1 is then calculated from equation (2) and the value a from equation (3), with K_2 0·001 min.$^{-1}$. These values are then inserted in equation (1) and the theoretical curve is generated.

RESULTS

During the course of this work it became apparent that the rate of disappearance of neuraminic acid in 0·1 N acid at 80° as measured by the thiobarbituric acid reaction did not follow first-order kinetics. This is also true if any of the other colour reactions for this substance (given in the Experimental section) are used. The data plotted on a logarithmic scale are given in Fig. 1. When the neuraminic acid is estimated after separating it from the reaction mixture by absorption on to and elution from an anion-exchange column (acetate form) or is estimated directly by the thiobarbituric acid reaction, the apparent rate coefficient decreases from $1{\cdot}1 \times 10^{-3}$ min.$^{-1}$ initially to about 6×10^{-4} min.$^{-1}$ after some 20 hr., the greater part of the curvature being in the first few hours. With the direct Ehrlich's or resorcinol methods there is no apparent destruction during the first hour (there may even be an apparent increase), the rate of disappearance of reactive material slowly increasing thereafter. *N*-Glycolloylneuraminic acid behaves in the same manner as the *N*-acetyl compound. Changes in initial concentration of neuraminic acid within the range 50–1000 μg./ml. do not significantly affect the shape of the curves in Fig. 1.

The acid destruction of neuraminic acid was investigated by passing a sample that had been heated at 80° for 6 hr. in 0·1 N acid down a column of anion-exchanger in its acetate form. The unchanged neuraminic acid is adsorbed on to the column, the aqueous eluate giving very little colour in the thiobarbituric acid analytical procedure. The resorcinol and direct Ehrlich's reactions, however, were strongly positive. Mild alkaline treatment of this eluate (see Experimental section) regenerates considerable amounts of thiobarbituric

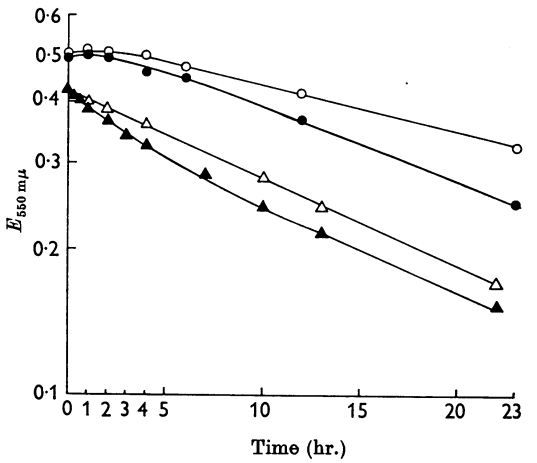

Fig. 1. Rate of destruction of neuraminic acid in 0·1 N-H_2SO_4 at 80°. The abscissa is logarithmic. ○, Direct Ehrlich's method; ●, direct resorcinol method; ▲, thiobarbituric acid method or resorcinol method after column separation; △, thiobarbituric acid method after adjusting to pH 7·5 and maintaining at 37° for 4 hr.

acid-reactive material (Fig. 2). Thus a neutral compound, which reverts readily to neuraminic acid, is formed from neuraminic acid under the influence of acid. This neutral compound, like the neuraminic acid from which it is formed, reacts with resorcinol and with dimethylaminobenzaldehyde but, unlike neuraminic acid, it gives very little or no colour in the thiobarbituric acid reaction. That the formation of this compound is responsible for the anomalies in the curve for destruction of neuraminic acid in acid is apparent, since if samples withdrawn from a solution of neuraminic acid in acid maintained at 80° are adjusted to pH 7·5 and incubated at 37° for 4 hr. before estimation with thiobarbituric acid, a good first-order plot for the destruction rate is obtained (K $6{\cdot}2 \times 10^{-4}$ min.$^{-1}$).

The equation describing the destruction of neuraminic acid in acid is given by Gibbons (1962*a*). It is not possible to use this exact expression to derive an equation analogous to (1) for the behaviour of the second member of a decomposition series when it decomposes in this manner. To obtain theoretical hydrolysis curves for the substances investigated for comparison with their observed behaviour, an average decomposition rate of 1×10^{-3} min.$^{-1}$ over the first 4 hr. has been used. Table 1 shows that the error involved in so doing is not serious but becomes so if longer hydrolysis times are considered. In most cases the liberation of neuraminic acid by acid from substances containing it is very rapid compared with the rate at which the liberated neuraminic acid is destroyed; thus changes in the value of $e^{-K_2 t}$ in equation (1) are small compared with those in $e^{-K_1 t}$ over the short period considered.

A selection of materials has been studied (see the Experimental section). Chemical analysis of the purified substances is given in Table 2. The goat serum and stroma preparation are not included in the Table since the only relevant figure is the neuraminic acid content, which was 25·2 mg./100 ml. of goat serum and 1·02% for the stroma preparation. In both essentially all (> 95%) of the neuraminic acid was split off by neuraminidase. The two bovine substances have been examined physically and are essentially homogeneous by the criteria used, though somewhat polydisperse. The bovine cervical mucopolysaccharide has been prepared as reported earlier (Gibbons, 1959) and presumably is of molecular weight in the region 4×10^6. The sedimentation constant and intrinsic viscosity imply a molecular weight $3{\cdot}23 \times 10^5$ for the submaxillary mucoprotein, assuming random-coil configuration. A molecular weight calculated from an interpolated sedimentation coefficient and the single diffusion constant observed is in good agreement with this value. It is discordant with the value $2{\cdot}34 \times 10^5$ that result sif a rigid ellipsoid configuration is assumed. A partial specific volume of 0·65 ml./g. has been assumed. This molecular weight may be compared with 9×10^5 for sheep submaxillary mucoprotein (Gottschalk & McKenzie, 1961) and $4{\cdot}2 \times 10^6$ for a preparation of bovine submaxillary mucoprotein (Bettelheim, Hashimoto & Pigman, 1962) isolated in a somewhat different manner. The other mucosubstances are less well characterized but chemical analysis does not suggest gross contamination. The neuramin-lactose is probably not pure, though it is analytically satisfactory. The remaining two materials studied are

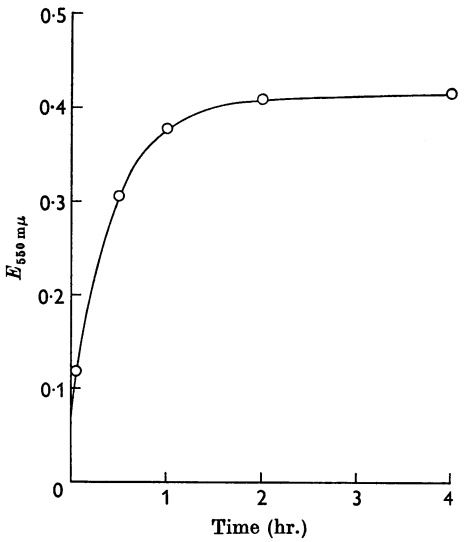

Fig. 2. The reappearance of neuraminic acid, as measured by the thiobarbituric acid reaction, in that portion of the products of neuraminic acid degradation in acid (0·1 N-H_2SO_4, 80°, 6 hr.) not retained by the acetate form of a strong anion-exchanger.

Table 1. *Comparison of observed and calculated rates of hydrolysis of neuraminic acid*

The observed destruction rate of neuraminic acid in 0·1 N-H_2SO_4 at 80° is compared with that calculated assuming a first-order reaction constant of 0·001 min.$^{-1}$. Observed values are means (10 experiments) ± S.D., with S.D. given as % of initial value, not of observed value.

Time of hydrolysis (hr.)	Theoretical value (% of initial)	Observed value (% of initial)
0·5	97·0	95·9 ± 1·06
1	94·1	93·1 ± 1·35
2	88·6	87·6 ± 1·80
4	78·6	78·5 ± 2·20
7	65·7	67·2 ± 1·51
12	48·7	54·0 ± 2·01
24	23·7	33·5 ± 2·19

Table 2. *Chemical characteristics of the mucosubstances studied*

	N (%)	Hexosamine (% as base)	Hexose (anthrone reaction, % as galactose)	Fucose (%)	Neuraminic acid (% as *N*-acetyl compound)			*O*-Acetyl (%)	Neuraminidase-labile neuraminic acid (% of total neuraminic acid)
					Direct Ehrlich's reaction	Thiobarbituric acid reaction (1 hr., 0·1 N-H_2SO_4, 80°)	Glycolloyl (%)		
Bovine cervical mucopolysaccharide	5·95	25·9	32·8	2·8	18·0	10·0	3·95	< 0·1	47·0
Bovine cervical mucopolysaccharide after alkaline treatment (0·05 N-Na_2CO_3, 100°, 20 min.)	5·90	26·3	29·2	2·8	17·4	9·7	—	—	47·5
Bovine cervical mucopolysaccharide after acid treatment (0·1 N-H_2SO_4, 80°, 50 min.)	6·25	25·8	31·7	0·3	5·0	2·1	—	—	< 1·0
Bovine cervical mucopolysaccharide after neuraminidase treatment	6·50	27·4	35·4	3·0	9·5	4·4	2·21	—	< 2·0
Bovine submaxillary mucoprotein	10·02	15·0	5·3	0·8	22·0	16·8	0·91	0·9	45·0
Bovine submaxillary mucoprotein after alkaline treatment (0·05 N-Na_2CO_3, 100°, 20 min.)	12·20	10·5	2·5	—	12·0	11·5	—	0·15	91·0
Bovine submaxillary mucoprotein after neuraminidase treatment	11·00	16·2	5·5	0·8	14·4	10·0	0·62	0·7	5·0
Human cervical mucopolysaccharide	6·11	20·0	27·0	11·0	8·8	8·0	0·11	—	75·0
Human bronchial mucopolysaccharide	6·31	19·1	21·9	12·5	9·1	8·3	< 0·02	< 0·1	83·0
Goat submaxillary mucoprotein	11·48	14·8	0·6	—	18·2	17·6	0·12	< 0·1	92·0
Pig submaxillary mucoprotein	9·88	10·0	8·0	1·1	24·1	15·5	3·95	< 0·1	90·0

mixtures. The bovine cervical mucopolysaccharide isolated from oestrous mucus is analytically similar to that isolated from pregnancy mucus, save that it contains rather less (14·9 %) neuraminic acid. It behaved in exactly the same manner as the pregnancy mucopolysaccharide, and only the latter therefore is recorded in the subsequent data. Hydrolysis curves (0·1 N-sulphuric acid at 80°) following the release of neuraminic acid by the thiobarbituric acid analysis have been obtained for all these preparations, and for the two bovine preparations after treatment with both alkali (0·05 M-sodium carbonate at 100° for 20 min.) and neuraminidase. The results for seven of the materials are indicated in Figs. 3–5, where the theoretical curve calculated as detailed in the Experimental section with the approximation just described is also shown. Of the materials for which figures are not given, all except two were essentially similar to that shown for goat serum, that is they liberated the maximal amount of neuraminic acid after approx. 50 min. and the observed points were close to the theoretical curve. The human bronchial mucopolysaccharide resembled rather more the human cervical mucopolysaccharide, and neuraminlactose, though agreeing fairly well with the calculated curve, showed maximal liberation of neuraminic acid after about 75 min., rather a longer period than that of most of the other substances.

Hydrolysis constants may be given for the scission of the neuraminosidic link in 0·1 N acid at 80° in those substances for which calculated and observed curves are concordant: for the neuramin-

Fig. 3. Neuraminic acid released by 0·1 N-H_2SO_4 at 80°, as measured by the thiobarbituric acid reaction. ○, Bovine submaxillary mucoprotein; ●, bovine cervical mucopolysaccharide. The theoretical curve, assuming a single first-order rate of hydrolysis, is drawn in each case; the experimental points are indicated.

idase-treated bovine cervical mucopolysaccharide, 0·016 min.$^{-1}$; for neuramin-lactose, 0·057 min.$^{-1}$; for the remaining substances approx. 0·1 min.$^{-1}$. However, the method of computation used depends upon the location of the time at which the amount of neuraminic acid is at its maximum value, and this cannot be defined to better than ± 5 min. The constants should therefore not be regarded as precise.

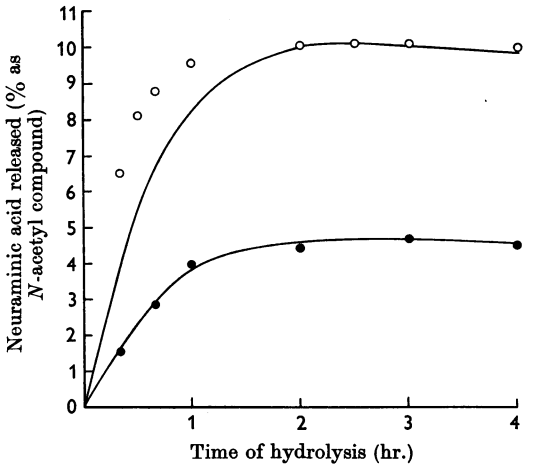

Fig. 4. Neuraminic acid released by 0·1 N-H_2SO_4 at 80°, as measured by the thiobarbituric acid reaction. ○, Bovine submaxillary mucoprotein after treatment with neuraminidase; ●, bovine cervical mucopolysaccharide after treatment with neuraminidase. The theoretical curve, assuming a single first-order rate of hydrolysis, is drawn in each case; the experimental points are indicated.

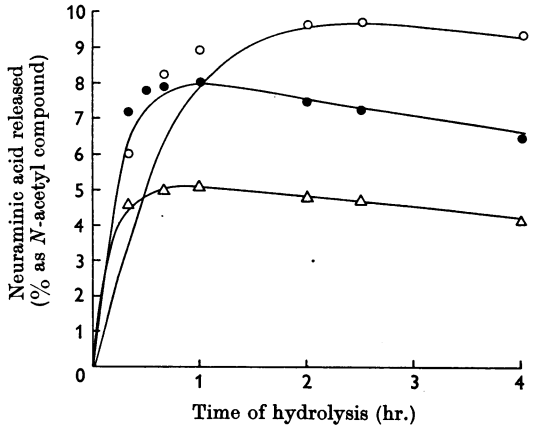

Fig. 5. Neuraminic acid released by 0·1 N-H_2SO_4 at 80°, as measured by the thiobarbituric acid reaction. ○, Bovine cervical mucopolysaccharide after alkaline treatment; ●, human cervical mucopolysaccharide; △, goat serum, diluted 1:5 (the amount of neuraminic acid released is expressed as mg./100 ml. of diluted serum). The theoretical curve, assuming a single first-order rate of hydrolysis, is drawn in each case; the experimental points are indicated.

The hydrolysis curve for the two undegraded bovine substances was also followed by the alkaline Ehrlich's procedure after column separation as described by Svennerholm (1958). In both cases the amounts released were apparently larger than found with the thiobarbituric acid reaction (*N*-acetylneuraminic acid being used as reference standard), with the cervical mucopolysaccharide considerably so. The maximum amounts released agreed fairly well with the total amount present, as estimated by the direct resorcinol or Ehrlich's reaction on the undegraded substances. As compared with the thiobarbituric acid reaction, the time at which maximal liberation of neuraminic acid occurred was decreased for the submaxillary mucoprotein to 75 min. whereas for the cervical mucopolysaccharide it was increased somewhat to about 3 hr.

Treatment with neuraminidase as described in the Experimental section of all the substances investigated results in the rapid release of neuraminic acid during the first 8–10 hr. Thereafter there was usually a very slow rise over the next 40 hr., though in some cases this was inappreciable. The figure given in Table 2 for the amount of neuraminidase-susceptible neuraminic acid is that released after 22 hr.; after a further 24 hr. the figure obtained was in no case more than 5% higher than this and it is clear that neuraminidase action has virtually ceased by this time. Fully active neuraminidase was still present in all cases, as between 65 and 75% of the total neuraminic acid in goat submaxillary mucoprotein was liberated in a further 4 hr. if this substrate was added to the 46 hr. digests. The neuraminic acid that remained indiffusible after neuraminidase treatment accounted for the balance of the total neuraminic acid, 95–108% of this substance being recovered overall in the experiments described.

A sample of bovine submaxillary mucoprotein that had been treated with acid under similar conditions to those described for the bovine cervical mucopolysaccharide was found to contain only 2·8% of neuraminic acid. Of this some 20% was susceptible to neuraminidase. As can be seen from Table 2, however, the acid-hydrolysed bovine cervical mucopolysaccharide was almost completely resistant to neuraminidase.

Analyses of the material split off from bovine cervical mucopolysaccharide by dilute acid at 80°, after fractionation on a charcoal column, are given in Table 3. The amounts of the components for which analysis is given do not add up to 100%, but both fractions, particularly the first, contain some Ba^{2+} ions. The two major fractions (F 1 and

Table 3. *Analyses of diffusible material from the hydrolysis of bovine cervical mucopolysaccharide after fractionation*

Diffusible material obtained from 200 mg. of bovine cervical mucopolysaccharide was analysed after fractionation on a charcoal–kieselguhr (3:1) column.

	Yield (mg.)	Hexosamine (% as base)	Anthrone colour (% as galactose)	Fucose (%)	Neuraminic acid (% as *N*-acetyl compound) Resorcinol	Thiobarbituric acid (direct)	Glycolloyl (%)
Aqueous eluate (F1)	10	>1	0	0	60	32·4	9·5
10% (v/v) Ethanol eluate (F2)	10	5·5	32·5	20·0	48·5	27·1	7·9
25% (v/v) Ethanol eluate	3	—	—	—	16·5	9·1	—

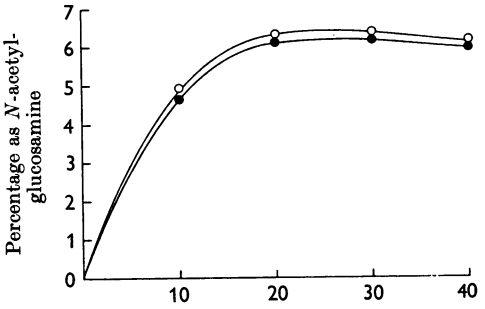

Fig. 6. *N*-Acetylhexosamine reaction: ●, before, and ○, after, treatment with neuraminidase. The amount of colour formed is expressed as a percentage of that given by *N*-acetylglucosamine heated in 0·05M-Na_2CO_3 for 4 min.

F2) after reduction with borohydride contain no material reactive in the direct Ehrlich's test; free neuraminic acid behaves similarly. The ketosidically linked neuraminic acid in neuramin-lactose and goat submaxillary mucoprotein is entirely unchanged by this treatment. The neuraminic acid in both fractions F1 and F2 is destroyed on treatment with 0·1N acid at 80° at the same rate as both authentic neuraminic acid and the neuraminic acid in the unfractionated diffusate. The 7-*O*-acetyl- and *N*-acetyldi-*O*-acetyl-neuraminic acid do not react in the thiobarbituric acid reaction (Aminoff, 1961) and when these are hydrolysed in 0·1N acid at 80° a curve similar to that of a mucosubstance results, i.e. the 7-*O*-acetyl group is hydrolysed at a similar rate to the neuraminosidic link. No material behaving in this way could be detected in the diffusible material from acid-treated bovine cervical mucopolysaccharide. Chromatography of fractions F1 and F2 (butan-1-ol–propan-1-ol–0·1N-hydrochloric acid; 1:2:1) revealed only *N*-glycolloylneuraminic acid with a trace of the *N*-acetyl compound. In another experiment in which the neuraminic acid was isolated in a different manner, two chromatographic components were found in ethyl acetate–acetic acid–water (3:1:3), of which the faster one coincided with *N*-glycolloylneuraminic acid. Material eluted from the chromatogram at positions corresponding to both spots contained only neuraminic acid and behaved as the unsubstituted compound when degraded with acid. This solvent requires a prolonged running time, and these papers were run twice, being dried in between. Experience has shown that double spots do sometimes arise under these conditions from authentic samples of neuraminic acid.

The *N*-acetylhexosamine reaction performed with the bovine cervical mucopolysaccharide and with the same material after treatment with neuraminidase gives results shown in Fig. 6. The curve for the neuraminidase-treated material is slightly above that for the undegraded substance, but when allowance is made for the loss of neuraminic acid from the neuraminidase-treated sample these curves may be considered to coincide.

DISCUSSION

Further evidence in favour of the reversible step proposed by Gibbons (1962*a*) in the decomposition process undergone by neuraminic acid in acid has been presented. This reversible step has necessitated an approximation in the calculation of predicted hydrolysis curves. The properties of the reversibly formed component strongly suggest that it is the (1→4)-lactone of neuraminic acid, a compound that has been prepared (Kuhn & Baschang, 1962*a*, *b*) though without description of its properties. The hydroxamic acid test for lactone linkages is not useful owing to the very dark colour of acid-hydrolysed neuraminic acid solutions.

Table 2 shows that the neuraminidase preparations used distinguish two types of neuraminic acid. Furthermore, it would appear that the neuraminidase-resistant neuraminic acid is also relatively more resistant to acid hydrolysis than that labile to this enzyme. Thus, where the pro-

portion of neuraminic acid that is neuraminidase-resistant is large (as in the two bovine mucosubstances), the materials behave on hydrolysis as though two different linkages are present that are being hydrolysed at different rates; the discrepant hydrolysis curves and prolonged time required before the concentration of neuraminic acid attains a maximum value are consistent with such a circumstance. The goat and pig submaxillary mucoproteins, the goat serum, the stroma preparation and the bovine submaxillary mucoprotein after alkaline treatment, although in some cases containing appreciable amounts of enzyme-resistant neuraminic acid, do not contain sufficient to alter significantly the hydrolysis curves, which have a maximum at about 50 min. The two human mucopolysaccharides, which contain a rather more substantial proportion of enzyme-resistant neuraminic acid, give slightly anomalous hydrolysis curves with a maximum at about 60 min. After removal of the enzyme-susceptible neuraminic acid with neuraminidase, the bovine cervical mucopolysaccharide gives a hydrolysis curve that agrees rather well with the theoretical one, though the rate of release of the free neuraminic acid is slow. Conversely, after removal of most of the acid-labile neuraminic acid with acid, the remainder is resistant to neuraminidase. Thus far considered the data suggest that these substances contain two different neuraminosidic linkages in varying proportions: one neuraminidase-labile with a hydrolysis constant (0·1 N acid at 80°) about 0·1 $min.^{-1}$, and the other with a hydrolysis constant about one-sixth of this value that is resistant to the enzyme.

Further results indicate that these considerations are not universally applicable. Inspection of the results obtained with bovine submaxillary mucoprotein after it has been degraded with acid, alkali or neuraminidase reveals that in this substance the source both of neuraminidase-resistance and anomalous behaviour on acid hydrolysis is not the same as that in the cervical mucopolysaccharide from the same species. Although the behaviour of the undegraded substances towards neuraminidase and acid (when the thiobarbituric acid analysis is used) is superficially similar, the two bovine substances on further examination are found to be contrasted in every other respect. Faillard (1959) has already reported that the neuraminidase-resistant neuraminic acid in bovine submaxillary mucoprotein is *O*-acetylated; this is confirmed, since alkali, which readily removes *O*-acetyl groups but to which the neuraminosidic link is rather resistant, renders the greater part of the neuraminic acid in this substance labile to neuraminidase. Alkaline treatment also removes anomalies in the hydrolysis curve. Bearing in mind that (*a*) 7-*O*-acetylneuraminic acid does not react in the thiobarbituric acid analysis, (*b*) the *O*-acetyl group is removed by acid at a rate comparable with that at which the neuraminosidic link is hydrolysed (this will presumably occur whether the neuraminic acid is free or not) and that (*c*) the column–resorcinol method of Svennerholm (1958) and the alkaline Ehrlich's method of Aminoff (1961) do, unlike the thiobarbituric acid method, estimate the free *O*-acetylated neuraminic acids, it can be seen that the *O*-acetyl groups could account for the observations on the bovine submaxillary mucoprotein that are here reported. The anomalous hydrolysis curves found for this mucoprotein are therefore presumably due not to the presence of intrinsically acid-resistant linkages, for Aminoff's alkaline Ehrlich's procedure shows that the neuraminic acid is in fact rapidly released, but to the release of part of the neuraminic acid in a form that must be further hydrolysed before it is detected by thiobarbituric acid. There is thus no reason why the neuraminosidic linkages should not be in the main of the type suggested by Gottschalk & Graham (1959).

It does not seem likely that the behaviour of the bovine cervical mucopolysaccharide can be accounted for in an analogous manner to that of bovine submaxillary mucoprotein, i.e. by assuming a substituent to be present in part of the neuraminic acid. Such a hypothetical substituent is clearly not alkali-labile (Fig. 5), and if it were acid-labile, acid-hydrolysed preparations of this substance should be in part neuraminidase-labile; and some anomalies would be expected in the hydrolysis curve of a neuraminidase-treated preparation (cf. the behaviour of the bovine submaxillary mucoprotein). It could be that such a substituent was resistant to the conditions of both acid and alkaline hydrolysis used; at one time it seemed possible owing to the low apparent yields of neuraminic acid (as measured by thiobarbituric acid) from acid hydrolysates of this substance that the neuraminic acid might be cleaved from the cervical mucopolysaccharide as a rather resistant dimer. Examination of the diffusible portion of an acid-hydrolysed specimen of bovine cervical mucopolysaccharide revealed no evidence of substituted neuraminic acids; furthermore all the neuraminic acid released by acid was found to have a free reducing group. An alternative explanation has now been found for the low apparent yields obtained with the thiobarbituric acid analysis.

A consideration of the behaviour of bovine cervical mucopolysaccharide in the *N*-acetylhexosamine reaction throws some light on the structure of the neuraminidase-resistant linkages. In an earlier report (Gibbons & Roberts, 1963), in which the effect of acidic hydrolysis and periodate oxid-

ation on this reaction were considered, it was shown that removal of the neuraminic acid residues by acid gave rise to a marked increase in the amount of reactive *N*-acetylhexosamine. This additional reactive amino sugar could be, unlike that in the undegraded substance, destroyed by periodate. It was pointed out by these authors that the presence of a (1→3)-linked hexosaminide chain, the terminal unit of which was substituted in position 4 by neuraminic acid, would be consistent with these observations. If this provisional structure be accepted, then it is the terminal *N*-glycolloylneuraminosyl-(2→4)-*N*-acetylhexosamine that appears to be relatively resistant both to acid and to neuraminidase. However, very little is known at present of the chemical structure of this class of mucopolysaccharide, and it is possible that other changes, at present unidentified, in addition to the release of free neuraminic acid, may be occurring under the influence of dilute acid. A terminal *N*-glycolloylneuraminosyl-(2→1)-*N*-acetylhexosamine linkage that Kathan & Winzler (1963) have suggested may be present in the erythrocyte virus inhibitor could also account for the rise in *N*-acetylhexosamine reaction after acid hydrolysis. The terminal (2→4)-linked structure of Gibbons & Roberts (1963) should therefore be regarded as provisional.

The bovine submaxillary mucoprotein is the only material studied in which *O*-acetyl groups may be detected by the procedure of McComb & McCreedy (1957). The author has previously stated that bovine cervical mucopolysaccharide gave a positive reaction under these conditions (Gibbons, 1959) but re-examination of a number of samples has shown that this was due to a laboratory error. The origin of neuraminidase-resistance, where present, in the other substances studied is not therefore the *O*-acetylation of the neuraminic acid. These resistant linkages need not be the same as the (2→4)-neuraminosyl linkages provisionally identified above; but in the two human mucopolysaccharides they probably are. Neuramin-lactose [a (2→3)-linked neuraminoside] is hydrolysed in acid somewhat more slowly than the other substances studied, the bovine materials excepted. Gibbons & Cheeseman (1962) found that it was rather slowly hydrolysed by neuraminidase also. This suggests that the rate of cleavage by both these agents depends on the position to which neuraminic acid is attached in the adjacent sugar residue. There are some reservations, however, about the purity of the preparation of neuramin-lactose used.

Finally there appears to be a large discrepancy between the total amount of neuraminic acid in bovine cervical mucopolysaccharide as measured by the direct resorcinol or Ehrlich's reaction and as measured by thiobarbituric acid after acid hydrolysis. This is not due to the presence of acid-resistant peptide-linked neuraminic acid such as has been reported (O'Brien & Zilliken, 1959; Whitehouse & Zilliken, 1960), as Svennerholm's column–resorcinol or Aminoff's alkaline Ehrlich's procedures show that it is in fact all released. This investigation has shown also that no significant amount of substituted or neuraminosidically linked neuraminic acid is released either. The discrepancy was finally traced to the low colour yield given by *N*-glycolloylneuraminic acid in the thiobarbituric acid reaction. Warren (1959) gives a figure of 88 % for the colour given by *N*-glycolloylneuraminic acid as compared with the *N*-acetyl compound and this figure is repeated by Whitehouse & Zilliken (1960). Aminoff (1961), however, gives 62·5 % with his modification of the thiobarbituric acid reaction, and it appears that this is a more accurate figure for Warren's procedure also. Thus a sample of *N*-glycolloylneuraminic acid prepared from pig submaxillary glands in this Laboratory, and an authentic sample kindly supplied by Professor E. Klenk, gave figures of 58·5 and 59·2 % respectively by weight compared with authentic synthetic neuraminic acid, or 61·3 % (average) on a molar basis. The sample of purified pig submaxillary mucoprotein showed a discrepancy of the same order as the bovine cervical mucopolysaccharide. This material (Table 2) contains chiefly *N*-glycolloylneuraminic acid (21·7 %), though a little (3·0 %) of the *N*-acetyl compound is present also (cf. Saito, 1959). Smaller, but significant, discrepancies occur with the bovine submaxillary mucoprotein. It is concluded that low yields of neuraminic acid as estimated by the thiobarbituric acid reaction after hydrolysis for 1 hr. may be due to one or more of four causes: (*a*) the presence of relatively acid-resistant linkages; (*b*) the presence of *O*-acetylneuraminic acids; (*c*) the presence of a large proportion of the neuraminic acid as its *N*-glycolloyl derivative; (*d*) interference in the direct Ehrlich's or resorcinol estimation by extraneous material, e.g. pyrroles or sugars. For highly purified material rich in neuraminic acid (*c*) is by far the most important source of disagreement. When the thiobarbituric acid reaction is used to assay neuraminic acid in a preparation not previously studied a glycolloyl estimation is really an essential concomitant, and the time of maximal neuraminic acid release should also be investigated. Such analyses should also theoretically be corrected for neuraminic acid destruction and incomplete hydrolysis by using equation (3).

This work shows that neuraminic acid, although it normally seems to be present as a terminal non-reducing unit, may be attached to mucosubstances by at least two different linkages, which differ in

their behaviour on acid or enzymic hydrolysis. This is exemplified by the bovine cervical mucopolysaccharide, and to a lesser extent by the two human mucopolysaccharides studied. Where the neuraminic acid is present as its *N*-glycolloyl derivative, as in the bovine cervical and pig submaxillary materials, low colour yields are obtained in the thiobarbituric acid reaction, and where the neuraminic acid carries *O*-acetyl groups, as in the bovine submaxillary mucoprotein, these residues are resistant to neuraminidase. The presence of 7-*O*-acetylneuraminic acid leads to the further complication that, though the neuraminosidic link may be readily cleaved by acid, the resulting free 7-*O*-acetyl compound does not react with thiobarbituric acid until the ester linkage has also been split. This can result in incorrect analyses if the usual 1 hr. hydrolysis period (Whitehouse & Zilliken, 1960) is employed.

SUMMARY

1. The decomposition of neuraminic acid by acid is accompanied by the reversible formation of a compound, probably the lactone, which renders this decomposition kinetically complex. Notwithstanding this complexity, theoretical hydrolysis curves may be obtained for the release and destruction of neuraminic acid during treatment of substances that contain it with 0·1 N acid at 80°.

2. Several such substances, notably bovine cervical mucopolysaccharide and submaxillary mucoprotein, have been examined. Large deviations from the theoretical hydrolysis curves have been observed with these two substances; two human mucopolysaccharides give slightly discrepant curves.

3. By examining the two bovine substances after treatment with dilute acid and alkali and with the enzyme neuraminidase it is concluded that the behaviour of the submaxillary mucoprotein can be accounted for by the well-known presence of a substantial proportion of the neuraminic acid in this substance as its *O*-acetyl derivatives. The *O*-acetyl groups confer a considerable degree of neuraminidase-resistance on this material.

4. The superficially similar discrepancies that arise in the behaviour of the bovine cervical mucopolysaccharide cannot be accounted for in this way, and it is necessary to postulate a neuraminosidic linkage in this substance which is intrinsically somewhat more stable in acid than that usually encountered. This linkage is also neuraminidase-resistant, and accounts for rather less than half of the total neuraminic acid in this substance.

5. A consideration of the behaviour of the bovine cervical mucopolysaccharide in the *N*-acetylhexosamine reaction allows the tentative identification of the resistant structure as *N*-glycolloylneuraminosyl-(2→4)-*N*-acetylhexosamine.

6. The other materials studied contain a rather small proportion of their neuraminic acid linked so as to be resistant to neuraminidase, and their hydrolysis curves do not seriously disagree with those predicted. The two human materials contain a somewhat larger proportion (17–25 %) so linked and give slightly anomalous hydrolysis curves.

7. The erroneous statement that bovine cervical mucopolysaccharide contains *O*-acetyl groups is corrected.

8. The discrepancy between the total amount of neuraminic acid in bovine cervical mucopolysaccharide estimated in the thiobarbituric acid analysis and that shown by other methods is traced to the low colour yield of *N*-glycolloylneuraminic acid, as compared with the *N*-acetyl compound, on reaction with thiobarbituric acid.

This work is part of a research programme on the chemistry of cervical mucus which is maintained by the Population Council Inc., Rockefeller Institute, N.Y., whose support the author gratefully acknowledges. Professor G. Blix kindly presented samples of 7-*O*-acetyl- and *N*-acetyldi-*O*-acetylneuraminic acid, and Professor E. Klenk a sample of *N*-glycolloylneuraminic acid. These gifts are acknowledged with many thanks. Dr Margaret Jackson and Dr Lynne Reid kindly collected the human material from their patients.

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Specific Metabolic Repression of Three Induced Enzymes in *Escherichia coli*

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It is well known that the synthesis of many induced catabolic enzymes in micro-organisms is repressed by glucose. Earlier studies on β-galactosidase formation in *Escherichia coli* have established the following facts (see Mandelstam, 1961, 1962). The glucose effect is not specific, and any compound that the cells can use as a source of carbon and energy can repress the formation of β-galactosidase, although it may not do so in usual conditions of growth, i.e. in a shaken flask culture. Any carbon-source metabolite will cause repression when the bacteria are starved of nitrogen or magnesium or a specific auxotrophic requirement, and the supply of carbon from the substrate concerned is in excess of what can be used for growth. The degree of repression produced by a compound is directly correlated with its effectiveness as a growth substrate. These results are consistent with the assumption that it is not glucose itself, but some metabolite derived from it, that is the active repressor (see Neidhardt & Magasanik, 1956; Mandelstam, 1961).

It has also been shown that the repressor that produces the glucose effect does so both in constitutive and induced synthesis of enzyme, and the effect is not reversed by inducer added even in large amounts (Mandelstam, 1962). It therefore appears to be entirely distinct from the repressor that has been postulated by Jacob & Monod (1961) as the cause of inducibility. For this reason we shall refer to it in this paper as the metabolic repressor. The term 'catabolite repression' has been used to describe the effect (Magasanik, 1961). Since the present experiments and previous work (Mandelstam, 1962) show that the repressor can be derived not only catabolically but also anabolically from relatively small molecules (such as lactate) we have preferred to use the more general term 'metabolic repression'.

The further problem that arises is whether a single metabolic repressor controls the formation of all enzymes that are subject to the glucose effect or whether there is a specific metabolic repressor for each. The latter interpretation would be consistent with the finding of Neidhardt & Magasanik (1957) that in *Aerobacter aerogenes* the glucose effect on histidase could be relieved under conditions where *myo*inositol dehydrogenase remained repressed.

We have examined β-galactosidase, D-serine deaminase and L-tryptophanase in *E. coli*. These are all inducible and subject to the glucose effect. The results show that metabolic repression is indeed specific and that the repressor in each case is one of the end products of enzyme action or some substance closely related to it. The more general aspects of these findings have been discussed by McFall & Mandelstam (1963).

METHODS

Organisms. The following strains of *E. coli* K12 were used: 58–161, which had a requirement for methionine. The bacteria were inoculated into a minimal medium (see below) without methionine, and a back mutant was selected that had lost the requirement. This revertant, 58–161 (met^+), was used in all subsequent work. The following galactose-negative strains (also K12) were kindly supplied by Dr J. Lederberg: W 3092 (lacking ATP–D-galactose 1-phosphotransferase, EC 2.7.1.6); W 3094 (lacking UDP-glucose–α-D-galactose 1-phosphate uridyltransferase, EC 2.7.7.12); W 3805 and 4221 (lacking UDP-glucose 4-epimerase, EC 5.1.3.2). These strains will be referred to as kinaseless, transferaseless and epimeraseless respectively. The wild-type was W 3110; *E. coli*