2-Carboxypyrrole: its Preparation from and its Precursor in Mucoproteins

By A. GOTTSCHALK
The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

(*Received* 9 *March* 1955)

Over the past six years this laboratory has been engaged in an investigation of the chemical composition and of the pattern of certain mucoproteins. Interest in this class of compounds was greatly stimulated by Burnet and co-workers when they discovered that a number of mucinous secretions of human and of animal origin inhibited haemagglutination by influenza virus, but irreversibly lost this capacity upon treatment with living influenza virus or with a soluble enzyme obtained from *Vibrio cholerae* cultures referred to as receptor-destroying enzyme (RDE) (for review see Burnet, 1952). Already the first attempts to purify the biologically active agent of the mucinous material left little doubt as to its mucoprotein character (McCrea, 1948). When a homogeneous mucoprotein inhibiting virus haemagglutinin at very low concentration (1×10^{-5} g./l.) became available (Tamm & Horsfall, 1952; Ada & Gottschalk, 1952), it was shown that interaction between the inhibitor and purified influenza virus B resulted in the release of a diffusible compound, thought to be an *N*-substituted *iso*glucosamine (Gottschalk, 1951). The diffusible split product gave a positive Molisch test, had reducing power (in alkaline medium) and decomposed with considerable humin formation on heating with dilute mineral acid; it coupled with *p*-dimethylaminobenzaldehyde to give a stable purple colour, even after prolonged heating with alkali, but failed to do so after treatment with mineral acid. A compound with the same properties was obtained when RDE instead of virus was used as the catalytic agent.

Though the isolation of the split product clearly demonstrated the activity of the viral enzyme and thus disposed of the claim (Bauer, 1949) that viruses are devoid of enzymes, the chemical identification was rendered difficult by the small yield. Relevant information, however, was gained by an investigation of the mucoprotein. The presence in the split product of a sugar residue and the sensitivity of the biological activity of the mucoprotein to treatment with periodic acid at low concentration (Burnet, 1948) suggested that the carbohydrate moiety of the mucoprotein contained the segment involved in the enzymic process. The urine mucoprotein was found on analysis to contain hexosamine, galactose, mannose and fucose in the molar ratio 8:6:3:1, the hexosamine fraction being a mixture of glucosamine and galactosamine (Gottschalk, 1952; Odin, 1952). The polysaccharide could be detached from the protein by alkali treatment and its molecular size was shown to be smaller than that of lysozyme (molecular weight 15000). This relatively small polysaccharide may be regarded as the prosthetic group of the urine mucoprotein, about 150 of them being attached to each molecule.

Acid hydrolysis of the prosthetic group was accompanied by the formation of considerable amounts of dark humin matter, indicating the location in the polysaccharide of the compound released from the mucoprotein by viral enzyme or RDE action. A compound with similar properties was first isolated in crystalline form from bovine submaxillary-gland mucin by Blix (1936) in a yield of 0·075–0·150 % and recently termed 'sialic acid' (Blix, Svennerholm & Werner, 1952). The reducing monobasic acid of unknown structure, with the probable composition $C_{14}H_{23}O_{11}N$ (but see Discussion) was characterized by strong humin formation and liberation of carbon dioxide on heating with mineral acid, by the purple colour it gave with Ehrlich *p*-dimethylaminobenzaldehyde reagent even without alkali pretreatment (direct Ehrlich reaction) and by a violet colour when treated with Bial's orcinol reagent. Klenk & Lauenstein (1952) prepared from haemagglutinin-inhibitory mucoproteins (human urine and bovine submaxillary-gland mucoprotein) the crystalline methoxy derivative of neuraminic acid, $C_{11}H_{21}O_9N$, isolated first from gangliosides (Klenk, 1941) and exhibiting some properties again similar to those of the split product. Kuhn & Brossmer (1954) obtained the crystalline methoxy derivative of lactaminic acid, $C_{11}H_{19}O_9N$, from cow colostrum, a compound resembling neuraminic and sialic acid in some of its reactions, and Trucco & Caputto (1954) prepared from rat mammary glands a substance, neuraminlactose, consisting of neuraminic acid and lactose. Finally it was observed that the various virus haemagglutinin-inhibitory mucoproteins, and more specifically the prosthetic group and the compound released from it by viral enzyme action, gave the

direct Ehrlich reaction (Odin, 1952; Gottschalk, 1953, 1954*a*).

These findings taken together directed our attention to a pyrrole derivative as the probable acid-labile, alkali-stable structure responsible for the direct Ehrlich reaction of the biological material tested. In a preliminary note the isolation and identification of 2-carboxypyrrole from the alkali hydrolysate of bovine submaxillary-gland mucoprotein were reported (Gottschalk, 1953). We have since duplicated the results with homogeneous preparations of bovine submaxillary-gland and human-urine mucoprotein, using an improved method of purification. The 2-carboxypyrrole thus obtained in a yield of about 0·05 and 0·03% respectively of the mucoprotein was found to be contaminated with about 1% of a second, not yet identified, pyrrole derivative. Separation of the two compounds was achieved by chromatography.

As was expected, the same imino acid could be prepared from the product liberated from the mucoprotein by enzyme action. In this case 2-carboxypyrrole only, i.e. free from contamination by the second pyrrole derivative, was obtained in a yield of 0·03–0·05% of the mucoprotein.

It was surprising to find that 2-carboxypyrrole is not preformed as such in the biological material, but derives from a labile precursor, probably of the 2-carboxyhydroxypyrroline type, stabilized within the natural framework by substitution of the hydroxyl group but becoming rearranged readily to 2-carboxypyrrole on release of the substituent (Gottschalk, 1954*b*).

This paper gives a detailed account of the isolation, purification and identification of 2-carboxypyrrole from two homogeneous mucoproteins and from the diffusible compound resulting from enzyme action on the mucoprotein substrate. The evidence for identification rests on the catalytic reduction of the isolated compound to 2-carboxypyrrolidine (proline) and on the identical chromatographic and spectroscopic behaviour of the compound and of authentic 2-carboxypyrrole. The paper also provides evidence for the presence in mucoproteins of a precursor which upon very mild and short alkali treatment is transformed into 2-carboxypyrrole.

Two claims about the isolation and identification of 2-carboxypyrrole from biological material have been made previously. Minagawa (1946) hydrolysed a purified preparation of yeast invertase with 25% sulphuric acid for 5 hr. at 100° and extracted an amino acid with ethanol from the neutralized and concentrated hydrolysate. The crystalline substance, termed 'minaline', exhibited the same physical and chemical properties, including a positive ninhydrin reaction, as proline, but gave a 'special spectrogram'. The author did not compare 'minaline' with an authentic sample of 2-carboxypyrrole. The published spectrogram of 'minaline' does not resemble that of authentic 2-carboxypyrrole, and its positive reaction with ninhydrin, as well as the use of 25% sulphuric acid in its preparation, justifies the statement that 2-carboxypyrrole was not the amino acid isolated by Minagawa. Hiyama (1949) treated bull sublingual-gland mucin with 10% (w/v) KOH for 10 hr. at 100° and isolated by an elaborate procedure from the hydrolysate a crop of crystals in a yield of 0·08% of the original material. By elementary analysis the compound was identified as 2-carboxypyrrole. Hiyama regarded the isolated imino acid as a degradation product by hot concentrated alkali of a completely substituted pyrrolidine having a glucosamine residue incorporated in its structure in such a manner as to share two carbon atoms with the amino sugar. It is not known whether pyrrolidines are transformed into pyrroles under the conditions applied, and Hiyama's results cannot therefore be interpreted. It would appear that the use by Hiyama of hot concentrated alkali in the preparation of 2-carboxypyrrole has obscured the realization that the pyrrole compound is not the degradation product of a stable saturated heterocyclic substance, but rather derives by rearrangement from a labile precursor of the hydroxypyrroline type. In our hands the lengthy procedure described by Hiyama is not a practical way to obtain crystalline 2-carboxypyrrole from mucoproteins.

MATERIALS

Preparation of bovine submaxillary-gland mucoprotein. Bovine submaxillary glands freshly collected at the abattoirs were immediately submitted to the procedure described by Curtain & Pye (1955). Purification in this procedure is achieved by methanol fractionation at low temperature in the presence of barium ions. The final mucoprotein solution was exhaustively dialysed against water and used either as such or after freeze-drying, storage *in vacuo* and reconstitution. In electrophoresis it moved as a single peak in phosphate buffer pH 7·0 and $I = 0{\cdot}2$.

Preparation of human-urine mucoprotein. The procedure of Ada & Gottschalk (1952) was followed.

Synthesis of 2-carboxypyrrole. Pyrrolylmagnesium iodide was prepared by Oddo's (1909) method, the optimum conditions developed for the Grignard reagent by Gilman & Meyers (1923) being applied. Dry CO_2 was passed through the ether solution of the compound. Instead of the resulting product being decomposed with H_2SO_4, an excess of N-NaOH was added, the mixture heated to boiling and the $Mg(OH)_2$ formed filtered off (McCay & Schmidt, 1926). After concentration and acidification with HCl pure, white crystals of 2-carboxypyrrole were obtained in satisfactory yield, m.p. 189–190°; on a filter-paper chromatogram the material ran as a single spot in all solvents tested.

Receptor-destroying enzyme (RDE). This was prepared from the filtrate of *Vibrio cholerae*, as described by Ada & French (1950).

METHODS

Paper partition chromatography. One-dimensional descending chromatograms were run on Whatman no. 1 paper strips (8 in. × 18½ in.) in glass jars of 10½ in. diameter and 20 in. height. Three μl. of the solutions were applied as individual spots along a line ruled 2¾ in. from the narrow edge of the strip. As markers authentic samples of known substances were run in a concentration approximating to that of the unknown, an adjustment usually requiring a preliminary run. For elution purposes the solution was applied in a 0·5 in. band along the starting line. The papers were inserted into a stainless-steel trough and held in position by two glass plates 8½ in. × 1½ in. No provision was made for constant temp.

The solvents were butanol–acetic acid–water (4:1:5, by vol.), butanol–pyridine–water (6:4:3, by vol.), *s*-collidine saturated with water and phenol saturated with water; in the last case a few crystals of KCN were added to the stationary phase of water saturated with phenol. Commercial *n*-butanol was rectified in a fractionating column and the fraction distilling at 117–118° collected and used. Phenol was purified by steam distillation and the two phases of the distillate were separated appropriately. Commercial *s*-collidine was purified as described by Partridge (1948). Pyridine, A.R. grade, was redistilled before use.

After the run the papers were dried at room temp. in a current of air for several hours. Chromatograms run on washed filter paper for elution of compounds were dried for 24 hr. or longer to ensure complete evaporation of the solvent. Elution was carried out in a glass chamber, as described by Aminoff & Morgan (1951).

Pyrrole derivatives are easily traced with an Ehrlich reagent prepared by dissolving 100 mg. *p*-dimethylaminobenzaldehyde in a mixture of 3·0 ml. conc. HCl and 3·0 ml. ethanol and adding 18·0 ml. *n*-butanol. The isatin spray contained 0·2 g. isatin in a mixture of 4·0 ml. acetic acid and 96·0 ml. *n*-butanol (Acher, Fromageot & Jutisz, 1950).

Spectrophotometry. A Hilger Uvispek photoelectric spectrophotometer and 0·5 cm. silica cells were used for the ultraviolet examination. The wavelength scale of the apparatus was calibrated by means of a mercury–hydrogen arc lamp.

Catalytic reduction of 2-carboxypyrrole. 2-Carboxypyrrole is reduced only with difficulty to 2-carboxypyrrolidine (proline). Following mainly Putokhin (1930) the procedure was as follows: 10 mg. platinum oxide (Adams's catalyst) and a mixture of 0·5 ml. acetic acid and 0·5 ml. ethanol was placed in a 25 ml. Erlenmeyer flask and, after removal of the air and equilibration with O_2-free H_2, shaken in H_2 for 2 hr. at room temp. 2-Carboxypyrrole (24·0 mg.) and 1 drop of a solution containing 6·3 g. $FeCl_3$/100 ml. water were added to the reduced catalyst and shaken in H_2 for 45 min. at room temp. Finally 0·2 ml. ethanol containing 0·19 g. HCl/ml., 1 drop of the $FeCl_3$ solution and another 10 mg. catalyst were added and the mixture was shaken for 3 hr. in H_2 at room temp. Occasionally H_2 consumption did not proceed with the material of biological origin. In this case a preliminary treatment of the material with the catalyst in order to adsorb to its surface poisoning substances, followed by removal of the catalyst, proved useful. At the end of the procedure the contents of the flask were filtered, 5 ml. water added and the mixture was dried in a desiccator over KOH and H_2SO_4. On chromatographic analysis of an ethanol extract of the dried material the presence of proline was established by the blue colour of a spot reacting with isatin and coinciding in R_F value and colour shade with an authentic sample of L-proline run as marker on the same strip. R_F values found for proline in the solvents mentioned and in the same order were: 0·31, 0·04, 0·15, 0·86.

Direct Ehrlich reaction. To test for the presence in mucoproteins and proteins of a component reacting directly with Ehrlich reagent 1 vol. of reagent was added to 5 vol. of a 2% (w/v) solution of the protein and the mixture heated at 100° for 10 min. In a positive test a stable purple colour soon appears, deepening on further heating. Reagent: 500 mg. *p*-dimethylaminobenzaldehyde were dissolved in a mixture of 5·0 ml. conc. HCl and 5·0 ml. water (Werner & Odin, 1952). Tryptophan did not produce a stable purple colour under these conditions.

Dry matter. A portion of the mucoprotein solution was dried at 92° and weighed.

Haemagglutinin-inhibitory activity of mucoprotein. This was determined as described by Burnet (1948), heated PR8 influenza virus being used as indicator.

RDE activity. This was determined as described by Burnet & Stone (1947).

Preparation of 2-carboxypyrrole from mucoproteins

(1) To 500 ml. of a 1% (approx.) (w/v) solution of the bovine submaxillary-gland mucoprotein were added 50 g. $Ba(OH)_2,8H_2O$, 10 ml. ethanol and 1·0 ml. octan-2-ol, and the mixture was refluxed in N_2 at 100° for 8 hr. After cooling, CO_2 was slowly bubbled through the hydrolysate and the precipitate formed was removed and washed with water. The pooled supernatant liquid and washings (pH 7·5) were concentrated under diminished pressure at 40° to a small volume, filtered and extracted with ether, and the ether was discarded. The aqueous phase was ice-cooled, acidified to pH 4·0 with N oxalic acid or with N-H_2SO_4 and the acid solution after removal of the precipitate extracted several times with ether, the pH of the aqueous phase being readjusted between extractions. The combined ether extracts were concentrated *in vacuo* and washed with water. After separation from the water phase the ether layer was thoroughly dried over anhydrous Na_2SO_4 and the ether evaporated *in vacuo* at low temp. The dried residue formed a snow-white, thin film covering the glass wall; on exposure to air it gradually assumed a reddish colour. On chromatographic analysis this material showed the presence of two compounds reacting directly with Ehrlich reagent, one coinciding with authentic 2-carboxypyrrole, the other being a faster-running substance of unknown structure. On repeated addition of water and drying *in vacuo* at 20° the faster-running compound, being somewhat volatile with water vapour, could be largely removed, the final product containing not more than 1% of the unknown contaminant. The average yield was of the order of 0·05% of the mucoprotein as determined spectrophotometrically, the highest yield obtained being 0·1%. This material was used for analysis by chromatography and for catalytic reduction. For spectrophotometric analysis an eluate of the spot coinciding chromatographically with authentic 2-carboxypyrrole was prepared. Earlier work was carried out with a preparation containing

about 7·5% of the contaminant; obviously for chromatographic analysis of the two compounds and for the spectroscopic analysis of their respective spots this was no disadvantage.

(2) Human-urine mucoprotein (2 g.) was treated in the manner described under (1). Average yield of 2-carboxypyrrole, 0·03%; contamination, as above (1).

Preparation of 2-carboxypyrrole from the diffusible product split from mucoprotein by enzyme action

(3) A solution (300 ml.) containing approximately 1% (w/v) bovine submaxillary-gland mucoprotein (virus haemagglutinin-inhibitory titre of solution, 85 000) was digested for 5 hr. at 37° (pH 7·0) with 1·0 ml. RDE solution containing 10^6 units enzyme, when the inhibitory titre was reduced to less than 100. The mixture was then dialysed against 10 vol. of distilled water for 48 hr. at 4° and the diffusate concentrated under diminished pressure at 40° to about 20 ml. Half of the concentrate was heated in three portions with alkali (0·1 N-Na_2CO_3, 0·1 N-NaOH and N-NaOH final concentration respectively) at 100° for a period of time (see Results) and then treated in the same way as under (1) but with omission of the last step. The yields of 2-carboxypyrrole were about 0·03% referred to the mucoprotein. The other half of the concentrate was not treated with alkali, but was otherwise submitted to the same procedure.

(4) A 1% (w/v) solution (100 ml.) of human-urine mucoprotein (inhibitory titre, 25×10^4), saturated with toluene, was digested with 1·0 ml. RDE solution (1×10^6 units) for 24 hr. at 37° (pH 7·0), i.e. for a much longer period than that required for reduction of the inhibitory titre to less than 100. The mixture was then dialysed against 10 vol. of distilled water for 48 hr. at 4°. Samples of the diffusate and of the non-diffusible material were taken and the intensities of their respective colour formation with Ehrlich reagent compared in a colorimeter; the result indicated a release by enzyme action of 55% of the substance reacting directly with Ehrlich reagent. The residual diffusate and the residual non-diffusible material were then processed as under (3) and (1) respectively, the conditions of alkali treatment in (3) being 0·2 N-NaOH final concentration, 100° for 40 min. The yield of 2-carboxypyrrole thus obtained from the diffusate (split product) was about 0·05%, and from the non-diffusible material (residual mucoprotein) about 0·01% of the total mucoprotein used.

RESULTS

Identification as 2-carboxypyrrole of the compound prepared from mucoproteins by alkali treatment

Colour reactions. Both synthetic 2-carboxypyrrole and the material prepared from bovine submaxillary-gland and human-urine mucoproteins reacted instantly in the cold with Ehrlich reagent, giving very similar purple colours; with both, the ninhydrin and Molisch reactions were negative.

Paper partition chromatography. Chromatography of the final products from the two mucoproteins revealed a purple spot when sprayed with Ehrlich reagent. This spot coincided in colour shade and R_F value with that given by authentic 2-carboxypyrrole. This acid will give a well-defined round spot only at low concentration; its application at higher concentration results in trailing. Only when a higher concentration of the final product was used was a faint faster-moving spot reacting directly with Ehrlich reagent detectable. At this stage of purification the ratio slow (2-carboxypyrrole) spot:fast spot was about 100:1, whereas in the neutralized hydrolysate before any further treatment this ratio approximated to 1:5. These ratios were obtained by assuming each compound to give the same colour yield and determining the minimum concentration necessary to give a detectable spot. Thus in the neutralized hydrolysate five times as much solution was required to detect 2-carboxypyrrole as for the contaminant (faster spot). The faster spot never trailed, and produced with Ehrlich reagent a colour very similar to that given by 2-carboxypyrrole. Spraying of the final product with aniline hydrogen phthalate and ninhydrin respectively did not result in the formation of any spots. When eluates of the two compounds reacting with Ehrlich reagent were prepared and rechromatographed separately, they ran as single spots with the same R_F values as before. Table 1 summarizes the R_F values in different solvents of the material obtained from submaxillary-gland mucoprotein. Nearly identical figures were found with the material prepared from urine mucoprotein. The higher R_F value given previously (Gottschalk, 1953) for the run in phenol could not be reproduced.

When synthetic 2-carboxypyrrole was submitted to the same chemical procedure as the mucoprotein (method 1) it did not change its chromatographic behaviour; after recovery from the ether extract it ran as a single spot with the same R_F value as before treatment. Freshly distilled, colourless

Table 1. *R_F values in various solvents of synthetic 2-carboxypyrrole and of two compounds present in a purified extract from submaxillary-gland mucoprotein*

Solvent	Synthetic 2-carboxypyrrole	Compounds of biological origin	
		Upper spot	Lower spot
Butanol–acetic acid–water	0·87	0·87	0·92
Butanol–pyridine–water	0·60	0·60	0·81
s-Collidine	0·58	0·58	0·71
Phenol	0·48 (trailing)	0·48 (trailing)	0·71

pyrrole did not lend itself to chromatographic investigation; owing probably to its volatility it could not be traced on paper after a run and appropriate treatment of the chromatogram. 2-Carboxypyrrole is very sensitive towards treatment with mineral acids. When the crystalline acid was heated in 0·4N-H_2SO_4 for 90 min. at 100° in a sealed tube, the H_2SO_4 removed as $BaSO_4$, the supernatant liquid concentrated *in vacuo* and run on paper, no Ehrlich-reacting spot was detectable.

Ultraviolet absorption spectrum. The ultraviolet absorption spectra of synthetic 2-carboxypyrrole and of the eluate of the spot obtained from submaxillary-gland mucoprotein and coinciding chromatographically with 2-carboxypyrrole are shown in Fig. 1. Between 220 and 290 mμ. the absorption curves are nearly coincident and their maxima are identical. The ultraviolet absorption spectrum of

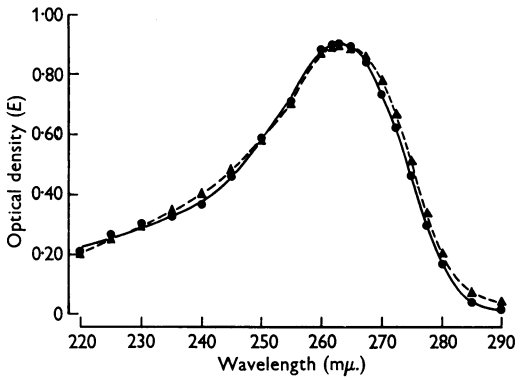


Fig. 1. Ultraviolet absorption spectra in ethanol of synthetic 2-carboxypyrrole and of the compound isolated from the alkali hydrolysate of bovine submaxillary-gland mucoprotein after removal of the contaminant. ●, Synthetic 2-carboxypyrrole; ▲, compound coinciding chromatographically with 2-carboxypyrrole.

the material obtained from urine mucoprotein was close to that of synthetic 2-carboxypyrrole, with maximum absorption at 263 mμ. In all these measurements the material was dissolved in ethanol.

Catalytic reduction. On catalytic reduction with Adams's catalyst (see Methods) the material isolated from submaxillary-gland mucoprotein gave rise to the formation of a substance which, in the solvents described, coincided chromatographically with authentic 2-carboxypyrrolidine (proline). No proline was detectable before reduction. When synthetic 2-carboxypyrrole in quantity equalling three times that of the contaminant present in the final product (see method 1) was submitted to catalytic reduction, the proline formed was undetectable by chromatography.

Molecular extinction coefficient of 2-carboxypyrrole

The ultraviolet absorption spectrum of synthetic crystalline 2-carboxypyrrole dried over P_2O_5 in a desiccator and dissolved in phosphate buffer (pH 7·0, $I = 0·05$) is given in Fig. 2. At 256 mμ. ($\lambda_{max.}$) $\epsilon = 11\,700$.

Ultraviolet absorption spectrum of untreated mucoprotein

In view of the high value of the molecular extinction coefficient of 2-carboxypyrrole and of the presence in submaxillary-gland and urine mucoprotein of the component (or components) responsible for the direct Ehrlich reaction in concentrations of 5–10% (w/w) (Odin, 1952; Klenk & Lauenstein, 1952) an attempt was made to trace

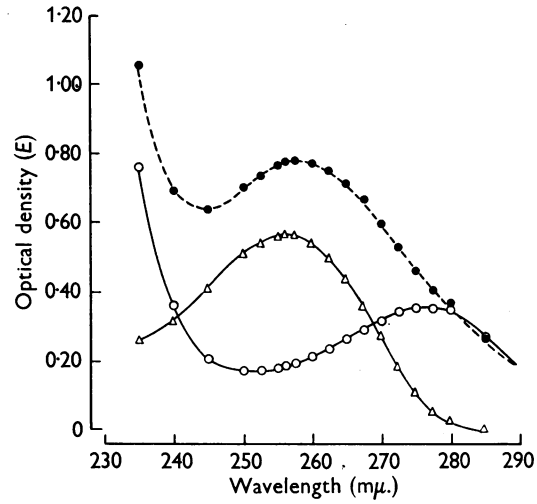


Fig. 2. Ultraviolet absorption spectra in phosphate buffer (pH 7·0, $I = 0·075$) of untreated 0·0435% (w/v) urine mucoprotein (○), 0·00115% (w/v) synthetic 2-carboxypyrrole (△), and a mixture of the two substances at these concentrations (●).

2-carboxypyrrole in the undegraded mucoprotein by its characteristic absorption maximum at 256 mμ. (in phosphate buffer). Since at low concentration the homogeneous urine mucoprotein provides a clear colourless liquid, a 0·087% (w/v) solution of this mucoprotein in phosphate buffer (pH 7·0, $I = 0·075$) was diluted with an equal volume of phosphate buffer and of a 0·0023% (w/v) 2-carboxypyrrole solution respectively and submitted to spectroscopic analysis. As may be seen from Fig. 2, no absorption peak at 256 mμ. is detectable in the untreated mucoprotein, whereas addition to the mucoprotein solution of 2-carboxypyrrole in a concentration amounting to 2·6%

(w/w) of the dry weight of the mucoprotein present resulted in a mixture exhibiting an ultraviolet absorption spectrum with an easily recognizable peak at 256 mμ.

The split product: transformation by alkali of a precursor into 2-carboxypyrrole

As with the untreated urine mucoprotein, spectroscopic analysis of the compound released from bovine submaxillary-gland mucoprotein by RDE at pH 7·0 and 37° failed to reveal the presence of 2-carboxypyrrole. However, after treatment of the split product with alkali under mild or more severe conditions (0·1N-Na_2CO_3 for 20 min.; 0·1N-NaOH for 45 min.; N-NaOH for 60 min.; 100°) a substance could be extracted with ether from the acidified solution which exhibited the ultraviolet absorption spectrum characteristic of 2-carboxypyrrole. Without preliminary alkali treatment no compound with any relevant absorption between 240 and 270 mμ. was obtained from the ether extract of the acidified solution (Fig. 3). On

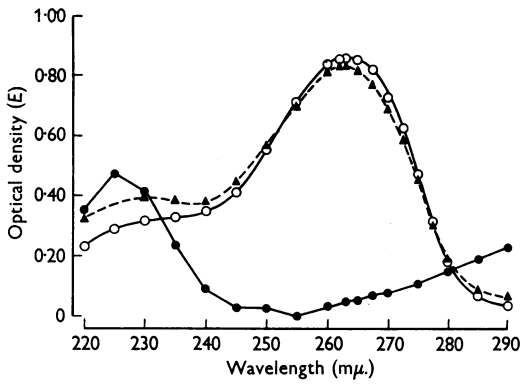


Fig. 3. Ultraviolet absorption spectra in ethanol of the ether-soluble material obtained from the alkali-treated product split off enzymically from bovine submaxillary-gland mucoprotein. ▲, 0·1N-Na_2CO_3, 20 min., 100°; ○, 0·1N-NaOH, 45 min., 100°; ●, no alkali treatment.

chromatographic analysis in the solvents described the compound obtained from the alkali-treated split product gave a single spot coinciding with synthetic 2-carboxypyrrole; the faster-running spot always present in the alkali hydrolysate of the complete mucoprotein was missing. No spot indicative of 2-carboxypyrrole was found in the untreated split product. The split product gave the direct Ehrlich reaction only on heating, whereas after alkali treatment the purple colour was formed instantly in the cold.

Analysis by chromatography and by light absorption in the ultraviolet of the material obtained by enzyme action on urine mucoprotein and subsequently treated with alkali (method 4) gave practically the same results as described in the preceding paragraph. The higher yield of 2-carboxypyrrole from enzyme action on urine mucoprotein (0·05 %) as compared with that from bovine submaxillary-gland mucoprotein (0·03 %) parallels the greater decrease in electrophoretic mobility with the former after RDE action (Curtain & Pye, 1955) under similar conditions.

Possible precursors of 2-carboxypyrrole in biological material

When the following substances were treated with 0·1N-Na_2CO_3 for 20 min. at 100°, none produced material reacting with Ehrlich reagent: glutamic acid, glutamine, glutamine amide, glutaminyl-asparagine, 2-carboxy-5-pyrrolidone, 2-carboxy-pyrrolidine, 4-hydroxy-2-carboxypyrrolidine (hydroxyproline). *N*-Acetylglucosamine, after this treatment, is known to give a purple colour with Ehrlich reagent (Morgan & Elson, 1934); however, the ultraviolet absorption spectrum of the compound or compounds formed bore no similarity to that of the 2-carboxypyrrole control treated in the same manner (Fig. 4), confirming previous results

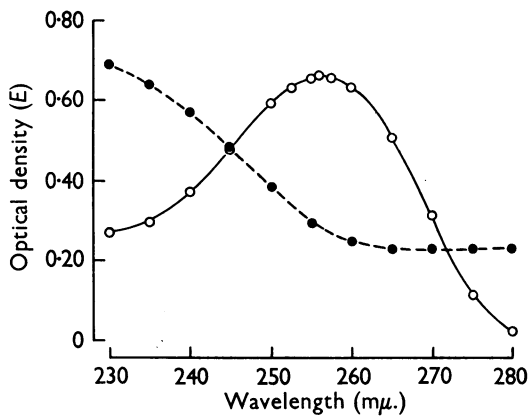


Fig. 4. Ultraviolet absorption spectra in 0·1N-Na_2CO_3 of *N*-acetylglucosamine (●) and of 2-carboxypyrrole (○) treated with 0·1N-Na_2CO_3 for 20 min. at 100°.

of Aminoff, Morgan & Watkins (1952). Chromatography, with butanol–pyridine–water as solvent, of the alkali-treated *N*-acetylglucosamine revealed a well-defined bluish-purple spot of R_F 0·64, fading after a few hours; the 2-carboxypyrrole control gave a reddish-purple spot of R_F 0·60, intensifying with time.

Distribution of the directly Ehrlich-reacting substance(s) in mucoproteins, mucopolysaccharides and proteins

Table 2 summarizes the results of testing 2% (w/v) solutions of various carbohydrate-containing and carbohydrate-free proteins and of an artificial mixture of galactose with a carbohydrate-free protein for the presence of a component or components reacting directly with Ehrlich reagent.

DISCUSSION

From the data presented it would appear that two preparations of 2-carboxypyrrole have been obtained from electrophoretically homogeneous mucoproteins (bovine submaxillary-gland and human-urine mucoprotein).

One preparation was obtained in a yield of about 0·05% of the mucoprotein after initial treatment of the protein with 0·64N barium hydroxide for several hours at 100°; this material was contaminated with about 1% of another substance closely related to 2-carboxypyrrole (see below). Separation of 2-carboxypyrrole from the contaminant was achieved by eluting the former from a chromatogram of the material run in an appropriate solvent.

The other preparation was obtained from the low-molecular-weight compound released from the mucoproteins by the *Vibrio cholerae* enzyme RDE and heated subsequently with mild alkali. This preparation proved to be chromatographically homogeneous, uncontaminated by the second substance; the yield was 0·03–0·05% of the mucoprotein. Owing to the low yield there was not enough uncontaminated material available to crystallize 2-carboxypyrrole in order to get a melting point or to have an elementary analysis performed. However, it is thought that the evidence for the structure of this compound is strong

Table 2. *Distribution of the directly Ehrlich-reacting substance(s) in proteins, mucoproteins and mucopolysaccharides*

Material	Molisch test	Direct Ehrlich reaction
Pepsin (twice cryst.)	Negative	Negative
Trypsin (twice cryst.)	Negative	Negative
Chymotrypsin (cryst.)	Negative	Negative
Insulin (cryst.)	Negative	Negative
Human globin (from haemoglobin)	Negative	Negative
β-Lactoglobulin (cryst.)	Faintly positive	Negative
Human-plasma albumin (five times cryst.), run 179, Dept. Phys. Chem. Harvard Medical School	Negative	Negative
Human-plasma albumin (five times cryst.) run 179, Dept. Phys. Chem. Harvard Medical School, plus galactose in a concentration of one-fifth of the protein present	Positive	Negative
Bovine plasma albumin (cryst.), Armour Laboratories, Chicago	Faintly positive	Faintly positive
Ovalbumin (cryst.)	Faintly positive	Negative
Human-urine mucoprotein (homogeneous)	Positive	Positive*
Human-sputum mucoprotein (homogeneous)	Positive	Positive*
Bovine submaxillary-gland mucoprotein (homogeneous)	Positive	Positive*
Sheep submaxillary-gland mucoprotein (homogeneous)	Positive	Positive*
Winzler's human-plasma mucoprotein (homogeneous)	Positive	Positive
Ovomucoid (purified)	Positive	Positive
Heparin (purified)	Positive	Negative
Hyaluronic acid (purified)	Positive	Negative
Chondroitin sulphuric acid (purified)	Positive	Negative

* 2-Carboxypyrrole isolated.

Interaction between 2-carboxypyrrole and reducing sugars in dilute mineral acid

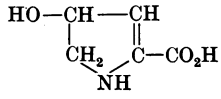
In Table 3 are recorded the colours formed by interaction of 2-carboxypyrrole and a reducing sugar under mild acid conditions. None of the reducing sugars produced any colour when 2-carboxypyrrole was omitted.

Table 3. *Colours resulting from heating an equimolar mixture of 2-carboxypyrrole (10 mg.) and a reducing sugar in 1·0 ml. 0·1N-HCl for 30 min. at 100°*

Sugar	Colour formed
—	Yellowish green
D-Glucose	Light brown
D-Galactose	Light brown
D-Fructose	Deep brown
Xylose	Deep brown
Ribose	Deep brown

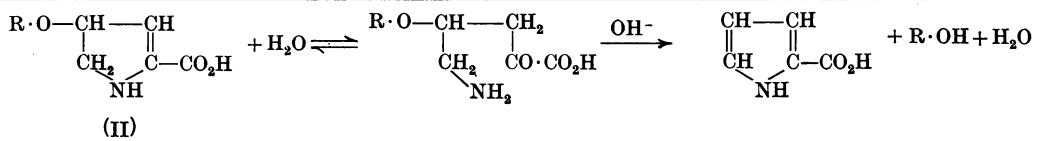
enough to permit its provisional identification as 2-carboxypyrrole. This evidence is as follows: chromatographic analysis in four different solvents of the pure material as obtained from mucoprotein and from the split product revealed coincidence in R_F values and colour shade in the Ehrlich reaction with an authentic sample of 2-carboxypyrrole. The ultraviolet absorption spectra of pure material from both sources practically coincided between 220 and 290 mμ. with that of authentic 2-carboxypyrrole. Catalytic reduction of the material from mucoprotein, which was contaminated with about 1% of another substance (see Results), caused the formation of a compound which on chromatographic analysis in four different solvents was found to be identical, in R_F value and in the blue colour produced by isatin, with authentic 2-carboxypyrrolidine (proline). The contaminant was excluded as the source of proline.

(Gottschalk, 1954*b*) the position 4 was assigned to the hydroxyl group because of the biogenetic relationship to hydroxyproline. 2-Carboxy-4-hydroxypyrroline (I) is a labile structure. It could therefore be assumed that within the framework of the mucoprotein and in the split product stabilization is effected by engagement of the hydroxyl group of (I) in a glycosidic linkage with



(I)

an adjacent sugar residue, the presence of which in the split product was previously shown (Gottschalk, 1951). The resulting structure (II) in aqueous solution will undergo reversible ring-opening (Fig. 5); the open-chain product, as a β-alkoxy-



(II)

Fig. 5.

On the other hand, the ultraviolet absorption spectra of the untreated native mucoprotein and of the product split off from the mucoprotein by RDE action (pH 7·0, 37°) indicate that 2-carboxypyrrole is not present as such in the biological material. The finding that 2-carboxypyrrole, though not present in the enzymically released product, is readily formed from it on such mild alkali treatment as heating at 100° for 20 min. in 0·1 N sodium carbonate narrows considerably the range of substances which may be assumed as possible precursors. Compounds of the pyrrolidine class, e.g. proline and hydroxyproline, are stable to alkali treatment. Glutamine on very mild alkali treatment undergoes cyclization by forming an amide bond involving the α-amino group at the expense of the preformed one, resulting in the production of 2-carboxy-5-pyrrolidone and ammonia (Vickery, Pucher, Clark, Chibnall & Westall, 1935). Treatment of 2-carboxy-5-pyrrolidone with barium hydroxide at 100° causes hydrolytic cleavage of the ring, with the formation of glutamic acid (Menozzi & Appiani, 1894). When heated with 0·1 N sodium carbonate none of these compounds produced a substance reacting with Ehrlich reagent in the cold.

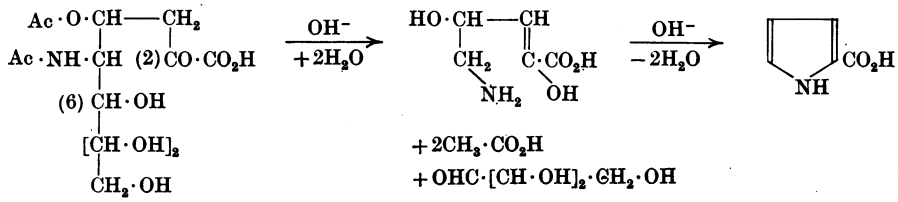
These negative results may suggest that the component of the mucoprotein and of the split product which on alkali treatment undergoes rearrangement to form 2-carboxypyrrole is a 2-carboxyhydroxypyrroline. In a preliminary note ketone, would be unstable to alkali (or acid), and reclosure of the ring concomitant with the expulsion of the sugar would yield 2-carboxypyrrole. The alkaline cleavage of glycosides of β-hydroxycarbonyl compounds is well known and the reaction mechanism is visualized as an elimination process rather than a hydrolysis (Ballou, 1954).

For the reason given an *O*-glycosidic linkage would appear to be more probable than an *N*-glycosidic linkage, as proposed earlier (Gottschalk, 1954*a*), when it was not known that 2-carboxypyrrole is not present as such in the mucoproteins.

When this paper was under review, a relevant note by Blix, Lindberg, Odin & Werner (1955) appeared. These authors give for sialic acid, a component of bovine submaxillary-gland mucoprotein (see Introduction), the provisional formula $C_{13}H_{21}O_{10}N$, H_2O and find the acid to contain an *N*-acetyl group, an *O*-acetyl group, a primary alcohol group, an α-hydroxy group and a total of five hydroxyl groups. On treatment with concentrated sulphuric acid carbon monoxide is liberated and one group can be titrated with hypoiodite (Willstätter–Schudel); there is no indication of the presence of a glycosidic linkage. If, in Fig. 6, (III) (or its pyranose form $<2, 6>$) is envisaged as a possible structure of sialic acid, the relationship between this acid, the unstable 2-carboxy-4-hydroxypyrroline (open form) and 2-carboxypyrrole becomes evident. Indeed Fig. 6 would seem to account for all the observations made so

far at Blix's and at our laboratory. It is understood that it is only a scheme; thus the elimination of the tetrose may occur during or after the alkali-induced cyclization to the pyrrole.

The relevant facts about the point of attack of the *Vibrio cholerae* enzyme on suitable mucoproteins are, first, that it was shown for the urine mucoprotein (Perlmann, Tamm & Horsfall, 1952) and for the submaxillary-gland mucoprotein (Curtain & Pye, 1955) that their net negative charge decreased after treatment with influenza virus or with RDE and that amino groups of pK value 10·8 to 11·2 were uncovered by RDE action on urine mucoprotein (Curtain, 1953). Secondly, it seems reasonable to assume that the rather strong basic group uncovered by RDE action and not counterbalanced by the loss in the split product of an equally strong basic group is masked in the native mucoprotein by an amide link with the carboxyl group of the precursor of 2-carboxypyrrole. However, more direct evidence is wanted to define RDE as an amidase.



(III)

Fig. 6.

Concerning the fast-running compound producing with Ehrlich reagent a colour very similar to that given by 2-carboxypyrrole the following data are available: (*a*) the compound is formed only when the complete mucoprotein is submitted to treatment with barium hydroxide at 100°; it is not obtainable from the enzymically released product or from synthetic 2-carboxypyrrole on alkali treatment. (*b*) Treatment of the compound even with 25 % KOH for 20 hr. at 100° does not produce 2-carboxypyrrole. (*c*) It has an absorption maximum in the ultraviolet very close to, if not identical with, that of 2-carboxypyrrole. (*d*) It has in aromatic solvents a much higher R_F value than that of 2-carboxypyrrole (Table 1). In butanol–pyridine–water the amide of 2-carboxypyrrole (kindly prepared by Dr J. W. Cornforth) has an R_F value identical with that of the faster-moving compound; however, the colours formed with Ehrlich reagent and the absorption maxima in the ultraviolet differ significantly with the two compounds. (*e*) The compound does not react with ninhydrin and is somewhat volatile with water vapour. (*f*) In the total alkaline hydrolysate of mucoprotein there appears to be five times as much of this compound as of 2-carboxypyrrole (see Results).

From these data we have formed the working hypothesis that treatment of the mucoprotein with barium hydroxide or stronger alkali affects the segment of the mucoprotein comprising the precursor of 2-carboxypyrrole and the adjacent base (see above) in a twofold manner. First, about one in six amide bonds linking the carboxyl group of the precursor with the adjacent base will be cleaved, and the precursor, after rearrangement as in Fig. 6, will yield 2-carboxypyrrole. Secondly, cleavage of the remaining amide bonds will be forestalled by the alkali-induced rearrangement (by cyclization) of the base in such a manner as to form an alkali-resistant compound with 2-carboxypyrrole. Obviously, the enzymic cleavage of the amide bond, followed by alkali treatment of the split product, could not result in the formation of the faster-moving compound and was not found to do so.

According to this hypothesis the low yield of 2-carboxypyrrole after alkali treatment of the mucoprotein would result, in part at least, from the formation of a new compound involving more than 80 % of the 2-carboxypyrrole present, just as the low yield of 2-carboxypyrrole from the enzymically released product is partly due to the fact that only a fraction of the total amount of precursor present is split off. In addition, in both cases, the loss of 2-carboxypyrrole inherent in the method of purification will be a contributing factor.

It seems of interest that the precursor of 2-carboxypyrrole is present in all virus haemagglutinin-inhibitory mucoproteins prepared so far in a pure state and, if the direct Ehrlich reaction under the conditions described (Methods) is indicative of the presence of 2-carboxypyrrole, also in human plasma mucoprotein and ovomucoid (Table 2). No such compound was detectable in crystalline proteins free from carbohydrate or in mucopolysaccharides containing hexuronic acid, nor did an artificial mixture of carbohydrate-free protein and galactose give a positive direct Ehrlich reaction.

SUMMARY

1. 2-Carboxypyrrole has been isolated from the alkali hydrolysate of homogeneous mucoproteins prepared from bovine submaxillary glands and from human urine. The imino acid, obtained in a yield of about 0·05%, was contaminated with approximately 1% of a substance of unknown structure. Separation of the two compounds was achieved by chromatography.

2. 2-Carboxypyrrole uncontaminated with the second compound, in a yield of about 0·03–0·05% of the mucoprotein, was obtained from the diffusible product released from the mucoprotein by the *Vibrio cholerae* enzyme (RDE) and treated subsequently with mild alkali.

3. Owing to its low yield the imino acid was not obtained crystalline. Its identification resides on the coincidence in chromatographic behaviour, in ultraviolet absorption spectrum and in proline production on catalytic reduction of authentic 2-carboxypyrrole and of the compound of biological origin.

4. The imino acid is not preformed in the biological material. Its precursor is, however, readily transformed into 2-carboxypyrrole when heated in 0·1N sodium carbonate solution for 20 min. at 100°.

5. Probable structures of the precursor are discussed.

6. The distribution of the precursor in proteins free of and associated with carbohydrate is described.

The author is greatly indebted to Dr Gertrude E. Perlmann, Rockefeller Institute for Medical Research, New York, Dr E. A. Kabat, Columbia University, New York, Professor R. J. Winzler, University of Illinois, Chicago, Dr J. F. McCrea, Yale University, New Haven and Dr J. Swan, Commonwealth Scientific and Industrial Research Organization, Melbourne, for generous gifts of crystalline proteins, purified mucopolysaccharides and glutamine derivatives. He also wishes to thank Professor A. J. Birch, University of Sydney, and Dr J. W. Cornforth, F.R.S., London, for profitable discussions on the subject and for their valuable advice.

REFERENCES

Acher, R., Fromageot, C. & Jutisz, M. (1950). *Biochim. biophys. Acta*, **5**, 81.

Ada, G. L. & French, E. L. (1950). *Aust. J. Sci.* **13**, 82.

Ada, G. L. & Gottschalk, A. (1952). *Aust. J. Sci.* **14**, 160.

Aminoff, D. & Morgan, W. T. J. (1951). *Biochem. J.* **48**, 74.

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.

Ballou, C. E. (1954). *Advanc. Carbohyd. Chem.* **9**, 59.

Bauer, D. J. (1949). *Nature, Lond.*, **164**, 767.

Blix, G. (1936). *Hoppe-Seyl. Z.* **240**, 43.

Blix, G., Lindberg, E., Odin, L. & Werner, I. (1955). *Nature, Lond.*, **175**, 340.

Blix, G., Svennerholm, L. & Werner, I. (1952). *Acta chem. scand.* **6**, 358.

Burnet, F. M. (1948). *Aust. J. exp. Biol. med. Sci.* **26**, 371.

Burnet, F. M. (1952). *Annu. Rev. Microbiol.* **6**, 229.

Burnet, F. M. & Stone, J. D. (1947). *Aust. J. exp. Biol. med. Sci.* **25**, 227.

Curtain, C. C. (1953). *Aust. J. exp. Biol. med. Sci.* **31**, 623.

Curtain, C. C. & Pye, J. (1955). *Aust. J. exp. Biol. med. Sci.* In the Press.

Gilman, H. & Meyers, C. H. (1923). *J. Amer. chem. Soc.* **45**, 159.

Gottschalk, A. (1951). *Nature, Lond.*, **167**, 845.

Gottschalk, A. (1952). *Nature, Lond.*, **170**, 662.

Gottschalk, A. (1953). *Nature, Lond.*, **172**, 808.

Gottschalk, A. (1954*a*). *Yale J. Biol. Med.* **26**, 352.

Gottschalk, A. (1954*b*). *Nature, Lond.*, **174**, 652.

Hiyama, N. (1949). *Tohoku J. exp. Med.* **51**, 317.

Klenk, E. (1941). *Hoppe-Seyl. Z.* **268**, 50.

Klenk, E. & Lauenstein, K. (1952). *Hoppe-Seyl. Z.* **291**, 147.

Kuhn, R. & Brossmer, R. (1954). *Chem. Ber.* **87**, 123.

McCay, C. M. & Schmidt, C. L. A. (1926). *J. Amer. chem. Soc.* **48**, 1933.

McCrea, J. F. (1948). *Aust. J. exp. Biol. med. Sci.* **26**, 355.

Menozzi, A. & Appiani, G. (1894). *Gazz. chim. ital.* **24**, 370.

Minagawa, T. (1946). *Proc. imp. Acad. Japan*, **22**, 130.

Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.

Oddo, B. (1909). *Gazz. chim. ital.* **39**, 649.

Odin, L. (1952). *Nature, Lond.*, **170**, 663.

Partridge, S. M. (1948). *Biochem. J.* **42**, 238.

Perlmann, G. E., Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 99.

Putokhin, N. I. (1930). *J. Soc. phys.-Chim. russe*, **62**, 2216. Cited in *Chem. Abstr.* (1931), **25**, 3995.

Tamm, I. & Horsfall, F. L. (1952). *J. exp. Med.* **95**, 71.

Trucco, R. E. & Caputto, R. (1954). *J. biol. Chem.* **206**, 901.

Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C. & Westall, R. G. (1935). *Biochem. J.* **29**, 2710.

Werner, I. & Odin, L. (1952). *Acta Soc. Med. Upsalien*, **57**, 230.