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Isolation of Ribonucleotide-Peptide Complexes from *Pasteurella multocida.** (29111)

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The presence of amino acids (or peptides) in ribonucleic acid preparations has been reported with RNA‡ from yeast(1-3), several microorganisms(4), pancreas(5), brain(6), liver(7), tobacco mosaic virus(7), etc. Nucleotide-peptide complexes have been detected in the TCA or ethanol extracts from *Chlorella*(8), yeast(9,10), etc. Similar observations are reported now with *Past. multocida.*

Materials and methods unless stated otherwise were the same as used previously(11). Dowex 1, 2% (X_2) 200-400 mesh and Dowex 50, 2% (X_2) 200-400 mesh were obtained from Bio-Rad Labs., Richmond, Calif., and Zeo-Karb 225 from Permutit Co. Ltd. L-*iso*-leucine-U-C^{14} (1.24 mc/mmole) and Na_2S^{35} (0.36 mC/mmole) were from the Radiochemical Centre, Amersham, Great Britain. The ninhydrin reaction was performed as described by Troll and Cannan(12).

Isolation and hydrolysis of RNA. The methods described in (13-15) were applied according to the following procedure. In steps 1-2 the amount of solvent used was 20 ml/g of cell residue. *Step 1 (removal of TCA-soluble fraction).* Acetone-dried *Past. multocida* (strain Beaufort No. 28 from Pasteur Inst., Paris) was suspended in distilled water and extracted with 5% (w/v) TCA at 4°. After centrifugation the residue was washed in the centrifuge with water, and then with ethanol (twice), ethanol-ether mixture (1:1; v/v) and ether. *Step 2 (removal of phospholipids).* The dried residue was extracted successively with *a*) ethanol-ether mixture (3:1; v/v) at 60° for 15 minutes, *b*) with methanol-chloroform mixture (1:1, v/v) at 65° for

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‡ Abbreviations used are: RNA, ribonucleic acid; TCA, trichloracetic acid; AMP, adenylic acid; CMP, cytidylic acid; GMP, guanylic acid and UMP, uridylic acid.

90 minutes and *c*) with ether. *Step 3 (extraction of RNA).* The dried residue was extracted successively at 100° for 1 hour each, 3 times with 10% (w/v) NaCl (10 ml/g of crude RNA) and twice at 90° for 15 minutes with 0.06 N $HClO_4$. The combined NaCl-$HClO_4$ extracts were poured on 2 vol of cold ethanol, and left overnight at 4°. The precipitate was collected by centrifugation and washed with ethanol-ether (1:1, v/v). *Step 4 (removal of protein).* RNA was dissolved by addition of N NaOH, the pH adjusted to 7, and the solution shaken for 10 minutes with chloroform-octanol mixture (4:1, v/v)(16, 17). Protein was removed by centrifugation and the treatment repeated until the separated chloroform was clear and the precipitate at the interface was negligible. RNA was precipitated from the aqueous phase by addition of 10 vol of acetic acid, collected by centrifugation and washed with ethanol-ether (1:1, v/v) until free of acetic acid. *Step 5 (hydrolysis).* RNA was digested for 16 hours at 37° with 0.5 N KOH (1 ml/0.1 g RNA). The solid residue was separated by centrifugation and washed in the centrifuge with 0.5 N KOH. To the combined supernatants were slowly added 10 N $HClO_4$, at 4° until pH 1 was reached. The precipitate was discarded by centrifugation and the supernatant brought to pH 7.5 with 5 N KOH. After 24 hours at 4°, the $KClO_4$ was removed by centrifugation and further KOH added until all $HClO_4$ had precipitated. After centrifugation the supernate contained the ribonucleotides.

Anion-exchange chromatography of RNA digest. The ribonucleotide mixture was resolved by chromatography on Dowex-1. A batch of resin (chloride form) was washed twice for 30 minutes, alternatively with N NaOH and N HCl, and then with distilled water until free of HCl. The resin was then packed in a 1 × 100 cm column. The nucleotide mixture, at pH 7.5, was applied to the exchanger and washed with water until the absorbancy of the effluent at 260 mμ was ≤0.010. Gradient elution of the nucleotides was effected by increasing linearly the acidity of the influent HCl solution. The HCl-system consisted of 2 chambers of equal cross sectional areas, containing the mixing chamber 0.0001 N HCl (pH 4) and the reservoir 0.01 M HCl (pH 2). Other experimental details were as described by Pontis and Blumson(18). The flow rate was 0.5 ml/min and 5 ml fractions were collected automatically. The effluent absorbancy was measured at 260 mμ.

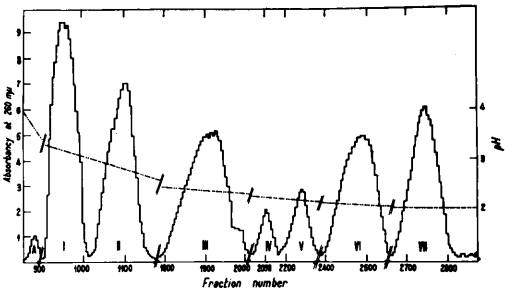

FIG. 1. Anion exchange separation of products of alkaline digestion of *Past. multocida* RNA. Experimental conditions described in text. 2.0 g of ribonucleotide mixture were loaded onto the column. Broken line: pH values. Solid line: absorbancy values. Peaks A-I, CMP; peaks II-III, AMP; peak IV, presumably AMP (plus nucleotide); peak V, UMP and peaks VI-VII, GMP.

Results and discussion. Fig. 1 shows the elute-gram obtained with RNA digest. The nucleotide sequence was similar to that obtained by Volkin and Carter(19) with rat liver RNA. CMP (peak I), AMP (peak II) and GMP (peaks VI and VII) were identified directly by their typical spectra at pH 2 and 7. With the isomeric AMP (peak III), absorbancies between 215-230 mμ were relatively lower with respect to the spectra of pure AMP. Fraction V yielded at pH 7 and 11 absorption spectra resembling that of UMP, with maxima at 260 mμ and minima at 230 mμ (pH 7) but absorbancies deviated from the corresponding typical spectra in the range of 215-230 mμ at both pHs. The eluates corresponding to the area under each peak were concentrated and subjected to paper ionophoresis(20) with 0.02 M citrate buffer solution (pH 5.8) and voltage gradient of 12-14 volt/cm (8 mA). A single ultraviolet absorbing spot was detected on the dried pherograms which overlapped with a ninhydrin-positive reaction. Control samples were eluted, the nucleotides adsorbed with

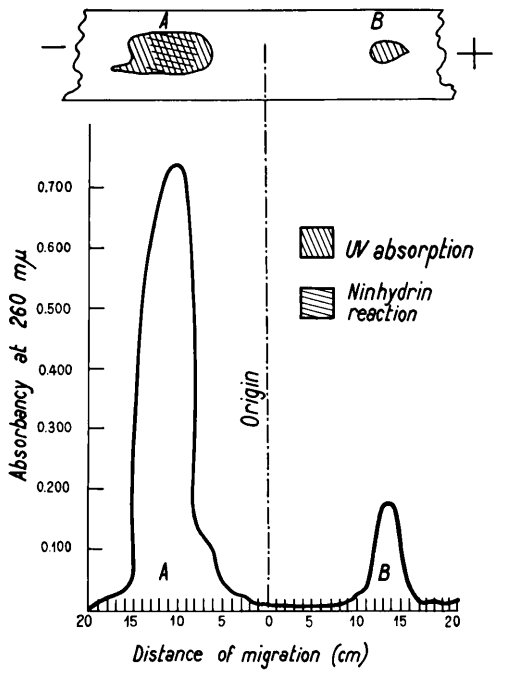

FIG. 2. Purification of a nucleotide-peptide complex from RNA of *Past. multocida*. Original material: fraction 2098 of Fig. 1. Ionophoresis on Whatman No. 1 paper for 10 hr at 12-14 volt/cm in 0.02 M citrate buffer, pH 5.8. Samples were eluted with distilled water, diluted 1:5 with 1.0 mM phosphate buffer at pH 7 and adsorbed with Norit A(21). After elution(22), absorbancy at 260 mμ was measured.

Norit A(21), eluted with pyridine(22), and the absorption spectra determined. Typical spectra were obtained with eluates from peaks A-I (CMP), II-III (AMP), V (UMP) and VI-VII (GMP). Samples from peak IV presented atypical spectra with maxima at 260 mμ (pH 2 and 7) and minima at 233 mμ (pH 2) or 226 mμ (pH 7) resembling AMP. Ionophoresis of peak IV (Fig. 2) separated one ninhydrin-negative component which moved towards the anode (like a nucleotide) and a ninhydrin positive one which moved towards the cathode and represented most of the original ultraviolet-absorbing material. Samples of the latter were subjected to *a*) paper ionophoresis at pH 5.8 with 0.02 M citrate buffer and *b*) paper ionophoresis at pH 8.8, with 0.02 M ammonium acetate buffer(23), then to cation-exchange chromatography on a column of 1.8 × 10 cm of Dowex 50, with the 0.01 N HCl-NaCl system(24), and finally to bidimensional paper chromatography with the phenol-water and butanol-propionic acid-water systems (25). The ultraviolet-absorbing substance accompanied systematically the ninhydrin-positive substance throughout the different analytical steps.

Further evidence for the nucleotide peptide association was obtained by introducing L-isoleucine-U-C^{14} as a label of peptides. Thus, a 10 to 1 culture of *Past. multocida* after 6 hour growth at 37° was added 0.5 mC of L-isoleucine-U-C^{14}. After 10 minutes agitation, the reaction was stopped with methanol (final concentration, 50% v/v) and the precipitate was collected by centrifugation and dried with acetone. To the dried-cells (4.7 g) was added the same amount of non-labeled material and RNA was extracted and hydrolyzed as described above. The nucleotides were subjected to anion-exchange chromatography and with each fraction measurements were made of *a*) absorbancy at 260 mμ, *b*) radioactivity caused by C^{14} and *c*) the ninhydrin reaction(12). The position of the maxima of the peaks detected by the 3 methods was coincident (Fig. 3) except peaks III and VI where the shape of curves corresponding to the ninhydrin reaction and radioactivity suggested the presence of highly radioactive peptides besides those associated with AMP and CMP respectively. The frac-

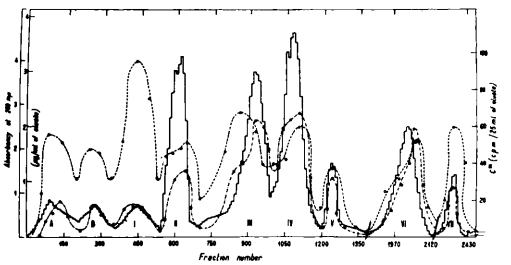

FIG. 3. Anion-exchange separation of products of alkaline digestion of *Past. multocida* RNA. Experimental details described in text for non-labeled RNA (Fig. 1). 1.8 g of ribonucleotide mixture was loaded onto the column. --○--, measurement of C^{14} (right ordinate); —●—, ninhydrin reaction (left ordinate: expressed as μg of glutamic acid/ml of eluate). Solid line without symbol, absorbancy at 260 mμ (left ordinate). Peaks A-B, unidentified; peaks I-II, CMP; peaks III-IV, AMP; peak V, UMP and peaks VI-VII, GMP.

tions eluted under each peak were combined, concentrated and purified by paper ionophoresis at pH 5.8 as above. The ultraviolet-absorbing (ninhydrin positive) material was eluted and hydrolyzed with 6 N HCl for 15 hours at 105° in a sealed tube. The digests were chromatographed bidimensionally on paper according to Benson *et al*(25) and the following amino acids were identified by the method of Sandua and Awapara(26) Peak II (CMP); cysteic acid, methionine, cystine, glutamic acid, aspartic acid, valine and proline. Peak III (AMP): cysteic acid, methionine, glutamic acid, aspartic acid, alanine, and glycine. Peak IV (AMP): cysteic acid, methionine, glutamic acid, aspartic acid and glycine. Peak V (UMP): cysteic acid, methionine, cysteine, glutamic acid, aspartic acid, glycine, histidine and proline. Peaks VI and VII (GMP): cysteine, methionine, homocysteine, glutamic acid, glycine, serine and valine. Cysteic acid is considered an artifact resulting from cysti(e)ne oxidation. Other amino acids were also revealed by the ninhydrin spray but because of their small amount were not identified (*iso*leucine belongs to this class).

Ribonucleotide-peptide complexes were also isolated from the 10% TCA-soluble extract of *Past. multocida*. On the basis of studies of Hase *et al*(8), incorporation of S^{35} into sulfur amino acids(27) was used to follow radiochemically the amino acid association with the nucleotides. Thus, to a 6 hour culture of *Past. multocida* was added Na_2S^{35} (1.1 mc/9 1) and further incubated at 37° for 1 hour. The culture was made to a 10% level (v/v) with respect to TCA and centrifuged in the Servall SS-1 centrifuge at 24,000 *g*. The supernatant was concentrated under vacuo at temperature $\leq 50°$ and TCA removed with ether. The extract was loaded onto a 4.5 $\times$ 30 cm column of Zeo-Karb 225 and the column was washed first with distilled water until glucose concentration in the effluent was $<1^0/_{00}$, and secondly with 0.01 N HCl and increasing concentrations of NaCl up to 0.05 N. Nucleotides were then eluted with 0.3 N HCl-0.05 N NaCl solution(24) and samples of eluate were concentrated, subjected to bidimensional paper chromatography(25) and radioautography. Several radioactive spots were obtained. The most active was submitted to paper ionophoresis at pH 5.8 as above. The ultraviolet-absorbing, single compound obtained reacted positively with ninhydrin and after elution and hydrolysis for 15 hours with 6 N HCl at 105°, yielded AMP, methionine, glycine, glutamic acid, aspartic acid and products of sulfur-amino acid degradation (taurine, cysteic acid, etc.).

The presence of peptides in preparations of RNA from *Past. multocida* does not seem to be conditioned to a certain method of preparation of RNA, as peptides remained attached to nucleotides after alkaline hydrolysis, paper ionophoresis, anion exchange and paper chromatography. Furthermore the chloroform-octanol treatment(16) is also a sensitive method for protein detection (1 part in 25000-40000) and therefore, its negativity indicated extensive deproteinization of the RNA extracted from *Past. multocida*.

The physiological significance of these facts is difficult to interpret in the light of the current hypothesis of protein biosynthesis(28) since messenger RNA would not itself bind amino acids at the time of the formation of peptide chains. However, Beljanski and Beljanski(29) have reported the fixing of "activated" L-amino acids by a rapidly labeled RNA containing both "messenger" and eosomal RNA, an observation consistent with the isolation of nucleotide-peptide complexes. The soluble nucleotide-peptide complexes can also be considered (as in other organisms(9,10)) fragments of peptide chains in process of formation.

Summary. RNA was extracted with 10% NaCl from acetone-dried cells of *Past. multocida* and deproteinized by treatment with the chloroform-octanol mixture. After alkaline hydrolysis, CMP, AMP, UMP and GMP were separated by anion exchange chromatography and paper ionophoresis. The nucleotides were tightly bound to peptide material. Nucleotide-peptide complexes were also detected in the TCA-soluble extract from *Past. multocida*.

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A Detailed Evaluation of Promazine Metabolism.* (29112)

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Unlike chlorpromazine and other phenothiazine tranquilizers which have been studied extensively over the last decade(1), promazine has received little attention as to its metabolism by man. Walkenstein and Seifter(2) reported that promazine is transformed by animals to yield nor$_1$promazine, promazine sulfoxide and nor$_1$promazine sulfoxide as urinary excretion products.† Additional products were also detected on their paper chromatograms but not identified. No evidence was found for didemethylation of the dimethylaminopropyl side chain. The possibility that promazine can undergo 3-hydroxylation as well as didemethylation was suspected from a more recent study(3), when promazine phenols were isolated whose properties proved to be analogous to the 7-hydroxychlorpromazine derivatives excreted during chlorpromazine therapy.‡

Confirmation of these preliminary indica-

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† The following abbreviations are used: TLC = thin-layer chromatography, P = promazine, CP = chlorpromazine, PSO = promazine sulfoxide, nor$_1$ = desmonomethyl, nor$_2$ = desdimethyl, PNO = promazine-N-oxide, HO = hydroxy, EdBzF = ethylene dichloride-benzene-formic acid-water (3/1/4/2), BuEt = n-butanol-ethanol-water (15/2/5) and BzA = benzene-acetic acid-water (2/2/1).

‡ Due to its symmetry, the 3- and 7-positions of promazine are equivalent (Fig. 1).