Identification of two major proteins of bovine pancreatic stones as immunoreactive forms of trypsinogens

Alain DE CARO,* Luc MULTIGNER and Henri VÉRINE

Unité de Recherches de Pathologie Digestive U 31 INSERM, 46 boulevard de la Gaye,
13258 Marseille Cédex 09, France

(Received 1 April 1982/Accepted 26 May 1982)

Two major proteins have been identified in sodium citrate extracts of bovine pancreatic stones from 15 glands with lithiasis. They were found to have a molecular weight of about 24 000 and were further characterized by a variety of methods, including polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, isoelectric focusing, two-dimensional electrophoresis, immunodiffusion, immunoelectrophoresis and determination of *N*-terminal residues. These two immunologically and electrophoretically different proteins were definitely shown to be immunoreactive forms of anionic and cationic trypsinogens, which are normal components of pancreatic juice. However, in contrast with both secretory trypsinogens, the stone proteins displayed an important charge heterogeneity under isoelectric-focusing conditions. A possible role for both secretory trypsinogens in pancreatic lithogenesis is suggested by the reproducibility of the data. Finally, two minor proteins with a lower molecular weight (about 11 000–13 000) have also been found to be present in all extracts, but have not yet been identified.

Pancreatic lithiasis, a disorder known to affect man and cattle (Vérine, 1973), is characterized by the presence of calcified stones (calculi) in pancreatic ducts. Human pancreatic lithiasis, also referred to as chronic calcified pancreatitis, is mainly associated with chronic alcohol consumption in Western countries and to malnutrition during childhood in tropical countries (Sarles, 1974). Although the aetiology of bovine pancreatic lithiasis remains unknown, the animal model may be considered as a good approach to the study of human disease (Vérine, 1973).

In previous studies it has already been shown that human and bovine pancreatic stones are mainly (95% by weight) composed of calcium carbonate (Périnet *et al.*, 1972), and X-ray-diffraction studies demonstrated the presence of CaCO₃ in the form of calcite (Mallet-Guy *et al.*, 1969). Also carried out was a preliminary investigation of the mechanism(s) governing CaCO₃ formation in pancreatic juice (Moore & Vérine, 1975) and the search for an organic matrix in pancreatic stones, since the

importance of matrices in various calcifications has been repeatedly stressed (Slavkin, 1972).

In this laboratory it was fairly recently shown that a major protein does exist in human pancreatic stones (De Caro *et al.*, 1979). This protein, a single polypeptide chain with a molecular weight of 13 500, was found to be immunologically identical in up to ten different cases and present in either normal or pathological pancreatic juice, though it has not yet been identified.

The paucity of material yielded by human pathology prompted us to study bovine pancreatic stones, which are more abundant (Vérine, 1973). Moreover, from the point of view of comparative mechanism(s) of calcification, the organic part of bovine pancreatic stones has an interest of its own, since bovine and human pancreatic stones have a similar mineral composition (Périnet *et al.*, 1972).

In the present study the two major proteins from bovine pancreatic stones were characterized as immunoreactive forms of cationic and anionic trypsinogens. As in man, these proteins are also present in normal pancreatic juice.

Materials and methods

Materials

Bovine pancreatic stones were collected in Danish

Abbreviations used: Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Mes, 4-morpholine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

* To whom reprint requests should be addressed.

and French slaughterhouses. In the present study, 15 cases were used.

Pure bovine pancreatic juice, devoid of free proteolytic activity, was kindly provided by Dr. L. Sarda (Université de Provence, Marseille, France) as a freeze-dried powder.

Bovine trypsin and chymotrypsinogen A were purchased from Worthington (once- and five-times-crystallized respectively) and bovine trypsinogen (once-crystallized) was from Merck.

Bovine chymotrypsinogen B and anionic trypsin were kindly supplied by Dr. O. Guy (U31 INSERM, Marseille) and Dr. A. Puigserver (CBM, CNRS, Marseille) respectively.

Commercial bovine trypsin was further purified by affinity chromatography on Kunitz pancreatic trypsin inhibitor–Sephacrose. A total of 8000 trypsin units were loaded on a 1.5 cm × 9 cm column equilibrated with 10 mM-Mes buffer, pH 7.0, containing 50 mM-CaCl₂ and 200 mM-NaCl. After the column had been washed with 50 mM-CaCl₂/200 mM-NaCl, trypsin was eluted by the same solution adjusted to pH 1.2. The protein was desalted by filtration on Sephadex G-25 equilibrated with 1 mM-HCl, and freeze-dried.

Trypsin-free porcine enterokinase was provided by Opochimie, Monte-Carlo, Monaco.

Enzyme assays

Chymotrypsin and trypsin activities were measured with 10 mM-Ac-Tyr-OEt and Tos-Arg-OMe solutions as substrates respectively (Figarella, 1966).

When necessary, trypsinogen activation was performed with porcine enterokinase. In a typical experiment, 1.2 mg of stone protein was dissolved in 300 µl of 5 mM-Tris/HCl buffer, pH 7.8, containing 20 mM-CaCl₂ and 40 mM-NaCl and then incubated with the same volume of a solution containing 0.9 mg of enterokinase in the same buffer.

Enzymic units are given by the number of µmol of substrate hydrolysed/min, and specific activity is the number of trypsin units per mg of protein.

Method of protein isolation

As the first step, the stones were extensively washed for 6 days in 0.15 M-NaCl containing 0.05% NaN₃ under continuous magnetic stirring to remove contaminating superficial proteins, then dried by evaporation *in vacuo*. The dried stones were ground in a Danguomau mill (Prolabo). The resulting powder from individual cases was studied separately.

Proteins were repeatedly extracted from the stone powder at 4°C for periods of 5 days (up to nine times) under continuous magnetic stirring in a 10% (w/v) sodium citrate solution containing 0.05%

NaN₃. The suspension was then centrifuged (3000 g, 30 min) and the supernatant was filtered through a 0.2 µm-pore-size Sartorius membrane filter, then dialysed against distilled water by using semi-permeable dialysis tubing with a 3500-mol.wt. cut-off (Spectrum Medical Industries, Los Angeles, CA, U.S.A.). After another filtration, the dialysis residue was concentrated by freeze-drying.

Just before use, the resulting powder was again dialysed against distilled water to remove residual salts.

Protein determination

In all chromatographic procedures, proteins were detected by their *A*₂₈₀. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine trypsinogen as standard.

Production of antisera

Two rabbits were immunized with a total amount of 4 mg of antigen from an extract of different pancreatic calculi (cases 1 and 2). Each injection was given subcutaneously in the back skin and in the hindleg pads with 0.50 mg of antigen emulsified in complete Freund's adjuvant. Three booster injections were given at weekly intervals. After a month's rest, the whole procedure was repeated. Rabbits were bled at 1 week after the last injection. Hereafter antisera will be referred to as Ab-1 and Ab-2 (antibodies 1 and 2).

Monospecific antibodies against pure bovine cationic trypsin (Ab-Ti) were prepared by the same procedure, a total of 4 mg of antigen being used.

Immunological techniques

Ouchterlony double diffusion was performed on agar plates with 1.5% agarose A (Pharmacia) in 25 mM-veronal acetate buffer, pH 8.6, containing 0.03% NaN₃. When necessary, benzamidine (5 mM) was added to the buffer to prevent trypsinogen activation.

Immunoelectrophoresis was performed as described by Grabar & Williams (1953) for 25 or 120 min at constant voltage (10 V/cm).

Electrophoresis on agarose plates permitted the complete separation of the two stone proteins after 120 min.

Wide portions of agarose gel were cut out from the previously determined sites of migration of the two proteins, then reduced to small pieces from which the protein was eluted with distilled water.

The immunoelectrophoresis method of Osserman (1960), which permits the identification of a known protein among a mixture of proteins, was also used.

Plates were stained with 0.1% Coomassie Blue R 250, and destained with acetic acid/ethanol/water (2:9:9, by vol.).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis on 15% polyacrylamide gels was carried out in the presence of SDS as described by Laemmli (1970). A constant current of 3 mA/tube was applied and protein bands rendered visible by staining with Coomassie Blue R 250. Standard proteins used for molecular-size calibration included alcohol dehydrogenase (mol.wt. 37 000), pepsin (34 700), chymotrypsinogen A (25 700), trypsinogen (24 000), apomyoglobin (16 600), lysozyme (14 300) and cytochrome *c* (12 300).

Isoelectric focusing

Isoelectric focusing in polyacrylamide slab gels was performed with an LKB 2117 Multiphor apparatus. The gels contained 3.45% acrylamide, 0.15% *NN'*-methylenebisacrylamide, 8 M-urea, 1.6% Ampholine, pH 3.5–10, and 0.5% Ampholine, pH 2.5–4.0 (LKB).

Focusing was performed at a constant power for 2 h. For pI determinations, focusing strips were sliced and the profile of the pH gradient was established by measuring the pH of each slice. The gels were stained with Coomassie Blue R 250.

Two-dimensional gel electrophoresis

In the first dimension, isoelectric focusing was carried out as described above, and gels were then fixed, stained and destained as described by Jäcke (1979). Focusing strips of about 0.5 cm width were cut, soaked for 30 min in 30 ml of an equilibration buffer (10 mM-imidazole/1% SDS/1% β -mercaptoethanol, pH 7.0) and finally placed on an SDS/polyacrylamide slab gel covered by a 1.5% agarose A layer.

In the second dimension, the SDS/polyacrylamide-gel electrophoresis was performed on 15% acrylamide slab gels in 50 mM-imidazole/0.1% SDS buffer, pH 7.0, with an LKB Multiphor apparatus. Gels were run at constant current for 13 h and stained with Coomassie Blue R 250.

Carbohydrate analysis

The presence of sugars was investigated on SDS/polyacrylamide gels by periodic acid/Schiff staining. After electrophoresis, the gels were electrophoretically washed for 2 h to eliminate SDS in order to avoid 'artefactual' staining (Glossmann & Neville, 1971).

N-Terminal residues

N-Terminal residues were determined by dansylation as described by Gros & Labouesse (1969). The resulting dansyl-amino acids were identified by polyamide t.l.c. (Woods & Wang, 1967).

Results

Existence of two major proteins

The protein yield of repeated extractions was similar in all cases and estimated to be $0.10 \pm 0.01\%$ of the initial powder weight.

Analysis of the extracts by Ouchterlony double diffusion against Ab-1 and Ab-2 antisera showed two lines of precipitation in 13 out of 15 cases, and only one line in the two other cases. Moreover, a complete immunological identity between proteins from all batches was observed. After diffusion of the proteins from several extracts against either of the antisera, immunoelectrophoresis gave two lines of precipitation, namely a weakly anionic line and a cationic one. In all cases each protein exhibited the same electrophoretic mobility. Moreover, by short-time electrophoresis it was clearly established that the anionic and the cationic proteins are immunologically different (Fig. 1). The sensitivity of the

Fig. 1. Short-time immunoelectrophoresis of stone proteins

Four different samples of stone proteins were placed in the wells and the antiserum to bovine stone proteins was placed in the troughs.

immunoelectrophoresis technique is so good that both major proteins were always found to be present even when a single precipitation line was observed by the Ouchterlony method.

Molecular properties of the stone proteins

Molecular weight. After SDS/polyacrylamide-gel electrophoresis of extracts from different calculi, a major protein with mol.wt. 24 000 and two minor bands, corresponding to mol.wts. 13 000 and 11 000 respectively, were found in all cases (Fig. 2). With or without prior addition of β -mercaptoethanol, the same pattern was obtained, thus demonstrating that each protein contains a single polypeptide chain. Since the abovementioned immunoelectrophoresis assays clearly established the presence of two major proteins, whereas only one major component was observed on SDS/polyacrylamide-gel electrophoresis, additional experiments were performed in order to elucidate this point. Each stone protein was first isolated by electrophoresis on an agar plate and then submitted to a polyacrylamide-gel electrophoresis in the presence of SDS. Both experiments gave the same result, consistent with a molecular weight of about 24 000 for each protein.

N-terminal residues. After dansylation of the proteins and subsequent acid hydrolysis of the derivatives, N-terminal residues were identified as valine and phenylalanine respectively.

Carbohydrate content. A negative periodic acid/Schiff reaction on SDS/polyacrylamide gels demonstrated that extracted proteins were carbohydrate-free.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of stone proteins isolated from eight different cases (lanes 1-8). Lane 9 contains the standard proteins trypsinogen (top) and apomyoglobin (bottom) with mol.wts. 24 000 and 16 600 respectively.

Presence of these two major proteins in bovine pancreatic juice

Bovine pancreatic juice was tested by double diffusion against Ab-1 and Ab-2 antisera. In both cases two sharp lines of precipitation were observed.

Immunoelectrophoresis of bovine pancreatic juice against each immunoserum gave, as shown above for the stone proteins, one anionic and one cationic line of precipitation. It is noteworthy that juice and stone anionic proteins have a similar electrophoretic mobility, whereas the juice cationic protein migrated faster than the stone cationic component (Fig. 3).

Gel filtration of bovine pancreatic juice on Sephadex G-100 was performed in 10 mM-Tris/HCl buffer, pH 8.0, containing 500 mM-NaCl and 1 mM-benzamidine. The elution diagram showed two main peaks of protein. Peak I contained the high-molecular-weight ($\geq 50 000$) proteins.

Proteins eluted under peak II gave rise to chymotryptic and tryptic activities and therefore correspond to proteins with molecular weights close to 25 000. The effluents corresponding to these two fractions were pooled and each fraction was tested against Ab-1 antiserum by immunoelectrophoresis.

An immunoelectrophoretic pattern similar to that previously observed with either juice or stone proteins was obtained with peak II, which indeed displayed both an anionic and a cationic line of precipitation. These proteins present in juice and in peak II were found to be immunologically identical with the stone proteins (result not shown).

Identification of the stone proteins as cationic and anionic trypsinogens

Proteins with a molecular weight about 24 000 that are normally present in bovine pancreatic juice were tested against Ab-1 and Ab-2 antisera by Ouchterlony analysis. Cationic trypsinogen and cationic trypsin gave a precipitation line, whereas no result was obtained with either chymotrypsinogen A or B.

Fig. 3. Immunoelectrophoretic pattern of stone proteins (1) and of bovine pancreatic juice (2) against an antiserum to bovine stone proteins located in the trough

The presence of a possible immunoreactive contaminant in commercial preparations of trypsinogen and trypsin was ruled out, since the same precipitation line was also observed with a cationic trypsin sample highly purified by affinity chromatography. There was a complete immunological identity between the cationic trypsin(ogen) and one of the proteins extracted from the stones (Fig. 4a).

Further immunological experiments were then performed in order to study the identity between the cationic trypsin(ogen) and one of the proteins extracted from the stones. A complete immunological identity was observed between one of the two stone proteins and the cationic trypsinogen when tested against the immunoserum prepared with bovine cationic trypsin (Ab-Ti).

However, when tested with the same antiserum, only a partial immunological identity was observed between pure cationic bovine trypsin and the stone protein, implying that trypsin had additional antigenic determinants (not shown). Finally, when trypsinogen and the pure bovine cationic trypsin were allowed to react with Ab-Ti, a similar partial immunological identity was observed.

Identification of the second major protein as the anionic trypsin(ogen) was achieved by two different methods.

In the first one, when stone proteins and anionic trypsin were allowed to react against Ab-1 or Ab-2 antiserum, a complete immunological identity was observed between the enzyme and one of the stone proteins (Fig. 4a). In the second method, immunoelectrophoresis performed with the same material showed that the anionic stone protein and the anionic trypsin had similar electrophoretic mobilities (result not shown).

Moreover, immunoelectrophoresis of the stone proteins by Osserman's (1960) method clearly demonstrated the simultaneous identification of the two proteins: the cationic stone protein was found to be immunologically identical with the cationic trypsin(ogen), whereas the anionic stone protein was immunologically identical with the anionic trypsin(ogen) (Fig. 4b).

Conversion of stone proteins into active trypsins

On activation with enterokinase, stone proteins gave rise to enzymes hydrolysing a typical trypsin substrate (Tos-Arg-OMe), with a specific potential activity of 2 units/mg. Bovine cationic trypsinogen activated in the same conditions presented a specific potential activity of 300 units. Activation assays performed with two distinct samples of stone proteins led to the same conclusion.

Isoelectric point of stone proteins

Isoelectric focusing of stone proteins, which was performed for four different cases, showed two sets of bands. A cationic set contained five main bands with pI values of 6.5, 7.0, 7.5, 8.0 and 8.4, and an anionic set comprised at least four bands with pI values between 4.1 and 4.6. By use of the same technique, pI values of 9.2 and 10.1 were found for cationic trypsinogen and trypsin respectively, and 4.5 for anionic trypsin.

When the focusing strip was submitted to SDS/slab-gel electrophoresis (second dimension), we observed that all bands, either in the cationic or in the anionic set, migrated to the same position, which was similar to the position observed with cationic trypsinogen and anionic trypsin.

Discussion

The present paper describes the isolation, properties and identification of the two major protein components of bovine pancreatic stones. In addition, two minor proteins, with lower molecular weights (11 000–13 000 instead of 24 000), were also found to be present, but have not yet been studied.

Both major proteins extracted from stones provided by 15 different cases of bovine pancreatic lithiasis were found to be immunologically and electrophoretically different and to be easily separated by immunoelectrophoresis. They consist of a

Fig. 4. Immunological relationships between stone proteins and both cationic and anionic trypsins, characterized by the Ouchterlony method (a) and Osserman immunoelectrophoresis (b)

1. Cationic trypsin; 2. stone proteins; 3. anionic trypsin; S. antiserum to bovine stone proteins.

single polypeptide chain with estimated mol.wt. of 24 000.

The presence of two major stone proteins in bovine pancreatic juice has been established by the immunological reaction that exists between the whole pancreatic juice and antisera raised against the stone proteins. In pancreatic juice, the proteins that are responsible for this reaction were also found to have mol.wt. 25 000. Results derived from a number of immunological studies led us to the conclusion that the two stone proteins are immunoreactive forms of cationic and anionic trypsinogens. The lack of immunological identity between bovine anionic and cationic trypsinogens is in agreement with the markedly different amino acid compositions of the two zymogens (Louvard & Puigserver, 1974) and should be pointed out here.

A set of four different results may be considered as essential to establish the fact that the two stone proteins are trypsinogens rather than trypsin. (1) The immunoelectrophoretic mobilities of stone proteins are closer to those of both trypsinogens than to those of both trypsin, the latter being more basic than the former. (2) Determination of the *N*-terminal residues of stone proteins as valine and phenylalanine does agree with the already reported *N*-terminal amino acids of both trypsinogens. Valine and phenylalanine are known to account for 99% of the total amount of *N*-terminal residues (Davie & Neurath, 1955; Louvard & Puigserver, 1974; Webster & Offord, 1977). Furthermore, it is noteworthy that isoleucine, the *N*-terminal residue of all known trypsin, was absent. (3) By using an immunoserum raised against pure cationic trypsin, a complete immunological identity was observed between cationic stone protein and cationic trypsinogen, whereas the stone protein and cationic trypsin only showed a partial identity. (4) On activation by enterokinase, the two stone proteins displayed a weak, but characteristic, tryptic activity.

The two major stone proteins share with anionic and cationic trypsinogens several molecular properties, such as molecular-weight values, *N*-terminal residues and immunological properties, but have different isoelectric points. The anionic stone protein contains at least four components with pI values ranging from 4.1 to 4.6, in contrast with anionic trypsin, which gives a single band of pI 4.5 under the experimental conditions used in the present study. The heterogeneity observed for the cationic stone protein is still greater, since the protein was distributed over a wide range of isoelectric points (6.5–8.4).

This last result is in agreement with the observed immunoelectrophoretic mobilities, since the stone cationic line was longer and more acidic than that of native cationic trypsinogen (pI = 9.2). When bands isolated by isoelectric focusing were submitted to an

SDS/slab-gel electrophoresis (second run), they all exhibited the same (24 000) molecular weight, ruling out the possibility that the already-mentioned minor proteins might be still present.

The charge heterogeneity observed in the focusing patterns could not result from the use of urea, since freshly prepared solutions of high-grade urea were used and since the gels were freed from isocyanate (O'Farrell, 1975). Finally, the charge distribution cannot be due to the presence of sugars, as often described for other proteins (Reinhold *et al.*, 1968; Plummer & Sarda, 1973), since we found that the stone proteins were carbohydrate-free. The possibility of a difference in some amino acid substitution seems unlikely.

These charge differences might likely be due to a deamidation reaction, which results in a shift from negatively to positively charged proteins, as observed for the two stone proteins. In a review paper by Robinson (1974), a general electrophoretic heterogeneity caused by deamidation has been found in a large number of protein preparations. The instability of the amino acids glutamine and asparagine were related to changes in pH, ionic strength and temperature, and general properties of the solvent surrounding the amides could affect deamidation half-times. The stone proteins are soaked in an alkaline medium resulting from the presence of calcium carbonate. Under these conditions, deamidation could occur, especially if we consider the high amide content of the cationic and anionic trypsinogens [respectively 12.7 (Mikes *et al.*, 1966) and 7.3% (Louvard & Puigserver, 1974)], and the fact that these stones were kept at room temperature for several years (up to 11 years). However, it is not possible to know whether and how the deamidation reaction of trypsinogens occurs *in vivo*, and if this deamidation could play a role in the stone formation.

Formation of calcite concretions *in vitro* is a chemical event governed by supersaturation. However, pancreatic lithogenesis takes place in a protein-rich medium. Thus our findings regarding stone formation should be viewed with caution. It should be stressed that the protein content is identical in all the pancreatic stones studied and that we never detected the pancreatic secretory proteins known to have an affinity for calcium (phospholipase A₂, chymotrypsinogens). This reproducibility suggests that the presence of immunoreactive forms of trypsinogens is not fortuitous. The participation of specific proteins in the development of urinary stones has been recognized (Lian *et al.*, 1977). It is now necessary to determine whether these proteins function as nucleation or aggregation agents or whether they represent an inhibitor of mineralization. Experiments are now required to test these possibilities.

Bovine pancreatic stones were kindly provided by Dr. K. Velling (Randers, Denmark) and Dr. P. Renard (Caen, France). We gratefully acknowledge the sustained interest of Professor H. Sarles (U31, INSERM, Marseille). We thank Dr. D. Lombardo (U31, INSERM, Marseille) and Dr. A. Puigserver (CBM, CNRS, Marseille) for many stimulating discussions and for critically reading the manuscript. This work was partly supported by INSERM grant no. ATP-60-78-92.

References

- Davie, E. W. & Neurath, H. (1955) *J. Biol. Chem.* **212**, 515–529
- De Caro, A., Lohse, J. & Sarles, H. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1176–1182
- Figarella, C. (1966) *Bull. Soc. Chim. Biol.* **48**, 97–115
- Glossmann, H. & Neville, D. (1971) *J. Biol. Chem.* **246**, 6339–6346
- Grabar, P. & Williams, C. A. (1953) *Biochim. Biophys. Acta* **10**, 193–194
- Gros, C. & Labouesse, B. (1969) *Eur. J. Biochem.* **7**, 463–470
- Jäckle, H. (1979) *Anal. Biochem.* **98**, 81–84
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lian, J. B., Prien, E. L., Glimcher, M. J. & Gallop, P. M. (1977) *J. Clin. Invest.* **59**, 1151–1157
- Louvard, M. N. & Puigserver, A. (1974) *Biochim. Biophys. Acta* **371**, 177–185
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mallet-Guy, P., Perrin, M., Thozet, A. & Vérine, H. (1969) *C.R. Séances Soc. Biol.* **163**, 1838–1841
- Mikes, O., Holeysovsky, V., Tomasek, V. & Sorm, F. (1966) *Biochem. Biophys. Res. Commun.* **24**, 346–352
- Moore, E. & Vérine, H. (1975) *Gastroenterology* **68**, 959
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Osserman, E. F. (1960) *J. Immunol.* **84**, 93–97
- Périnet, G., Vérine, H., Lafont, R. & Haladjian, J. (1972) *C.R. Hebd. Séances Acad. Sci. Ser. D* **274**, 3303–3305
- Plummer, T. H. & Sarda, L. (1973) *J. Biol. Chem.* **248**, 7865–7869
- Reinhold, V. N., Dunne, F. T., Wriston, J. C., Schwarz, M., Sarda, L. & Hirs, C. H. W. (1968) *J. Biol. Chem.* **243**, 6482–6494
- Robinson, A. B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 885–888
- Sarles, H. (1974) *Gastroenterology* **66**, 604–616
- Slavkin, H. C. (1972) *The Comparative Molecular Biology of Extracellular Matrices*, Academic Press, New York
- Vérine, H. (1973) *Comp. Pathol. Bull.* **5**, 3–4
- Webster, D. & Offord, R. E. (1977) *Biochem. J.* **167**, 285–287
- Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370