Vol. 116, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1983 Pages 180-188

# CHARACTERIZATION OF RAT α1 MICROGLOBULIN

C. VINCENT, P. BOUIC and J.P. REVILLARD

Laboratoire d'Immunologie INSERM U.80, ERA 782 CNRS
Hôpital E. Herriot - Pavillon P
69374 LYON Cedex 08 - France

Received August 24, 1983

A molecule heterogenous in charge with an apparent MW of 30 000 d and associated chromophore and carbohydrate was isolated from rat urine. When compared to human α1-m its fluorescence spectra as well as staining pattern on agarose gel electrophoresis or electrofocussing were very similar. Furthermore, the specific antiserum prepared against the molecule detects an antigenic determinant also detected by monoclonal antibodies against human α1-m. For these reasons this protein can be considered as the rat equivalent of human α1-m.

Human α-1 microglobulin (H α1-m) was first isolated by Ekström and Berggard (1) in 1975 and has been described as a heterogenously-charged glycoprotein (2), present in urine and serum (3). Since then, other authors have reported the purification of apparently identical glycoproteins (4,5,6). However discrepancies still persist with respect to the cellular origin, tissue distribution and biological properties of H α1-m (7).

Studies of α1-m homologues in other mammalian species would not only be of interest from an evolutionary point of view, but, would also present a prerequisite for any experimental investigation on this molecule. So far, only guinea-pig α1-m has been characterized (8).

This report describes the isolation of rat α1-m (R α1-m) from the urine of rats treated with sodium chromate, a substance known to induce tubular reabsorption defects of low molecular weight (MW) proteins (9). Like guinea-pig α1-m, Rα1-m shares several biochemical and antigenic properties with its human homologue. Immunohistochemical studies on various rat tissues show an identical pattern of staining with these two reagents (Bouic et al., submitted for publication). Furthermore α1-m is recovered in similar positions after chromatography of rat or human serum.

## MATERIALS AND METHODS

**Urine and sera.** Seven week-old Wistar rats (150 to 200g) were treated weekly by intraperitoneal injections of sodium chromate 15 mg/kg body weight. The rats were kept in metabolic cages and the water supply was limited to 5 ml/day. Urines were collected each day, centrifuged at 20,000g and stored at -

0006-291X/83 $1.50

30°C in the presence of 0.015M sodium azide. Treatment and urine collection were continued during two months. Blood was collected by cardiac puncture and sera kept at -40°C until use.

**Preparation of antisera.** Antisera to rat α1-m were raised by injecting intradermally in 20 sites in the back of a rabbit 1 mg of pure α1-m diluted in 1 ml of saline and homogenized with an equal volume of Freund's complete adjuvant. After 4 weeks, a booster inoculation of the same amount of antigen in Freund's incomplete adjuvant was given intramuscularly. The rabbit was bled 10 days later. Serum was heat-inactivated (56°C, 30 min), and absorbed three times with human AB Rhesus positive pooled erythrocyte stromas, polymerized human IgG and B2 microglobulin. This procedure was used to remove rabbit natural antibodies. No absorption on rat cells or body fluids was performed in order to avoid the removal of specific anti- α1m antibodies.

Rabbit antisera and monoclonal antibodies against human α1-m were prepared as already described (10,11).

**Labeling of proteins.** α1-m was labeled with $^{125}I$ according to the method of Hunter (12) using 5 μCi for 1.25 μg of proteins and was then sampled and stored at -70°C prior to use.

**Polyethylene-glycol exclusion test (PEG-test).** Fifty μl of labeled antigen (20 ng/ml) was mixed with 50 μl of antibody dilution and 50 μl of buffer or cold antigen. All dilutions were done in RPMI 1640 Hepes buffer containing 10 % Fetal Calf Serum and 0.02 % $NaN_3$. After an incubation period of 12 h at 17°C, 1 ml of 10 % PEG 6000 in 0.05 M phosphate buffer (pH 7.5) containing 9.16 % Tween 20 and 0.015 M $NaN_3$ was aded to each tube. Five minutes later, the tubes were centrifuged at 2 500g for 15 min. at 4°C and the radioactivity in the pellet was counted.

**Gel chromatographic methods.** Salts, free pigments and peptides were eliminated from concentrated urine by filtration on Ultrogel AcA 202 columns (2.5 x 20cm), equilibrated with Tris-HCl 0.03M; NaCl 0.1M pH 8 and eluted at 5 ml/$cm^2$/h. These urines were then submitted to chromatography on Sephacryl S200 column (2.5 x 100 cm) in the same conditions as those described for the AcA 202 columns.

Chromatography on Ultrogel AcA 54 gel was performed on 1 x 5 cm columns equilibrated with the above-mentionned buffer containing 1 M guanidin hydrochloride. Calibration of the columns was carried out using human albumin (Calbiochem), Ovalbumin (Miles Laboratories), α-Chymotrypsinogen A, Cytochrome C, lysozyme (Sigma), Cyanocobolamin (Mercks): human IgG and B2m were prepared in our laboratory as already described (13).

**Affinity chromatography on Concanavalin A (Con A).** Hundred ml of Con A-Sepharose (Pharmacia) were equilibrated with Tris HCl 0.03 M, NaCl 0.1 M, CaCl2, MgCl2, MnCl2 1 mM, pH 8, in a 2.5 x 20 mm column. Inclusion of the protein solution was done at 2 ml/h, after which the column was washed at 15 ml/h with 10 volumes of buffer gradually supplemented with NaCl up to 1M. This was followed by elution of the fixed glycoproteins with 0.1 M αmethylmannoside and α-methylglucoside in the original buffer.

**Isoelectric focussing.** Isoelectric focussing on granulated gel (Ultrodex) with 2 % of Servalyte pH 3-6, was conducted with the LKB Multiphor apparatus following the manufacturer's instructions.

**Fraction analysis.** Concentration of urines and fractions were done by ultrafiltration under nitrogen pressure on YM 10 membranes (AMICON). Frozen urines were thawed, then centrifuged 20 min. at 48,000 g at 4°C prior to concentration. Protein content was measured by the Biorad protein assay using bovine albumin as standard. Electrophoretic analysis was performed in agarose

gels at pH 8.6 according to Jeppson et al. (14) and in polyacrylamide agarose gels in the presence of SDS (SDS PAGE) according to Laemmli (12.5 %) (15).

**Optical methods.** Fluorescence emission and excitation spectra were recorded with an Jobin et Yvon spectrofluo JY 3 spectrophotometer. A solution of 0.5 mg R α1-m/ml in 0.01M phosphate buffer pH 7.5 was used. The light absorption spectrum was determined in a Perkin-Elmer 550 spectrophotomeer with the same protein solution.

**Immunosorbent procedures.** Ig fraction was prepared from rabbit antiserum by dialysis against 0.02M acetate buffer pH 4.5 and subsequent filtration though DEAE Sepharose equilibrated with the same buffer. Ig were immobilized by overnight incubation at 4°C (10 mg/ml of gel in NaNCo3 buffer 0.1M O.5M NaCl pH 8.3) with CNBr activated Sepharose 4B.

## RESULTS

**Isolation of rat α1-m.** The isolation procedure were carried out with the hypothesis that R α1-m had biochemical properties comparable to those of H α1-m.

Two hundred ml of rat urine were concentrated then filtered on AcA 202 gel. Part of the dark brown colour was recovered with the proteins in the excluded volume, while the components which gave a certain viscosity and a characteristic odor to the concentrated urine were retarded in the gel. Urinary proteins, representing 98 mg, were fractionnated by chromatography on Sephadex S 200 gel. In the region delimited by the IgG and B2m markers, the eluate was separated into 6 fractions according to the optical density. Protein contents of fraction 1 to 6 were 18.4, 19.6, 30, 19.5, 3.5 and 1.0 mg, respectively. Analysis on SDS-PAGE indicated the presence of material with an apparent MW of 25-35,000d in fraction 3 and 15-35,000d in fraction 4. These fractions were pooled and chromatographed on Con A-Sepharose. After recycling, 12 mg of proteins remained unbound and 37.6 mg of glycoprotein could be eluted from the column. On SDS-PAGE the eluted fraction showed multiple bands, among which components with an apparent MW of 31,000d represented 9% of the material stained by Coomassie blue, as estimated by densitometric measures.

The fraction eluted from Con A-Sepharose was dialyzed against 0.1M glycin, then submitted to isoelectric focussing. Each fraction was analyzed on SDS-PAGE. Those recovered in the pH range 4.51 - 4.98 were found to migrate in the 30,000d zone. After neutralization and concentration, 6.3 mg of proteins with a brown colour were recovered.

Finally, after chromatography on AcA 54 gel most of the proteins were recovered as a sharp peak in a 60,000d position with a smaller peak around the 30,000d position (fig. 1). However, on SDS-PAGE the former 60,000d peak was resolved into a single band in the 30,000d position. The total protein content was 3.6 mg, representing 3.7 % of the initial material.

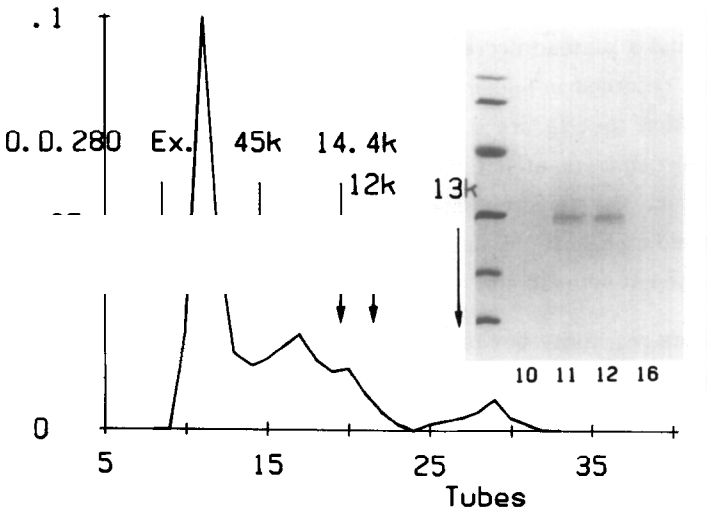


Figure 1 Chromatography of R α1-m on AcA 5-4 gel. The inset represents the SDS gel electrophoresis of the proteins in tubes 10, 11, 12 and 16. By increasing the volume of fraction analyzed in SDS an additionnal band at 70 000 d was observed in 11 and 12, and a band at 30 000 d in 16.

On agarose electrophoresis this fraction migrated in fast α-position and had a diffuse pattern of staining suggesting an heterogeneous charge (fig. 2). In view of the above-described characteristics, this fraction was considered as the rat equivalent of human α1-m and used for immunization of rabbit L 206.

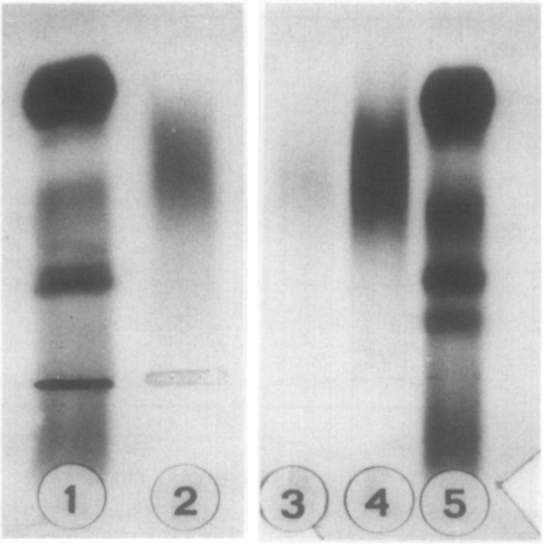


Figure 2 Agarose gel electrophoresis of purified R α1-m (2), H α1-m (3, 4), normal human serum (1, 5). Pure B2 microglobulin was mixed with human serum in 5.

**Fluorescence spectra.** The absorption spectrum showed a maximum at 278 nm and a plateau decreasing slowly from 300 nm onwards. No additionnal peak was recorded in higher wavelengths (fig. 3A). The fluorescence exitation and emission spectra are shown in fig. 3B. The excitation spectrum recorded with a light emission at 475 nm revealed 3 peaks of unequal intensity at 290,357 and 380 nm. The emission spectrum when the excitation is maintained at 290 nm, showed a peak at 330 nm. After excitation at 357 or380 nm, a broad peak was emitted at 470-480 nm.

**Cross-reactivity between rat and human α1-m.** The rabbit anti-R α1-m antiserum L 206 (10 mg total Ig/ml) was used in Ouchterlony's double immunodiffusion against concentrated rat urine (30 mg protein/ml) undiluted normal rat serum purified R α1-m (1 mg/ml), human urine (30 mg protein/ml) undiluted human serum and purified H α1-m (1 mg/ml) : no precipitation line could be detected.

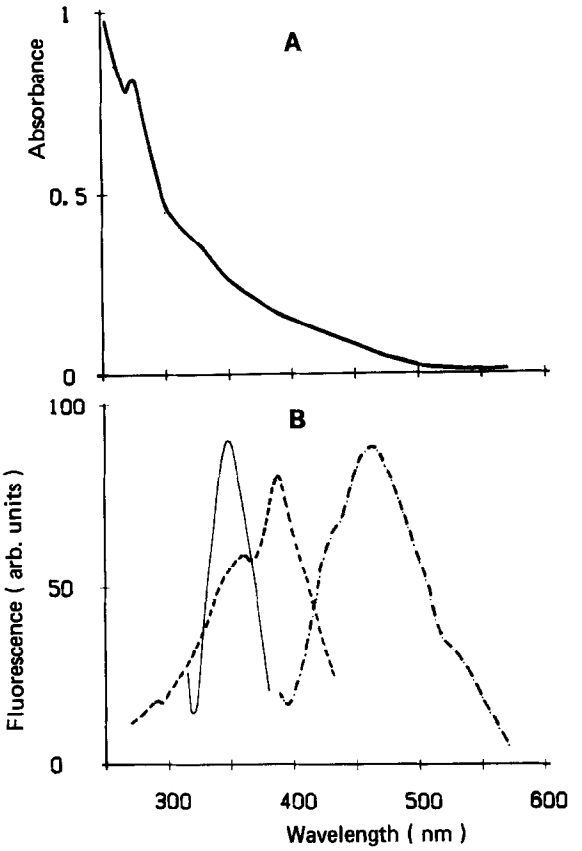


Figure 3 **A.** Absorption spectrum of rat α1-m between 250 and 600 nm. **B.** Fluorescence spectrum of rat α1-m. The excitation spectrum (— — —) was obtained by recording the light emission at 475 nm and the emission spectrum by exciting at 380 nm (—•—) or 290 nm (———). An amplification factor of 10 was used for the profile at 290 nm in comparison with the profile at 380 nm.

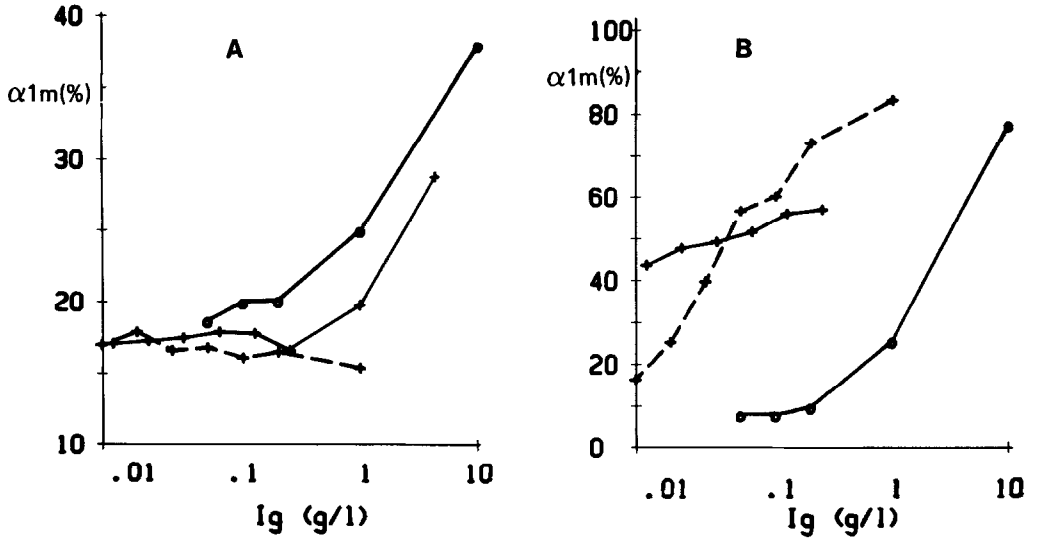


Figure 4 Binding activities of monoclonal antibodies H 23 (+——+), antiserum L 177 (+—+) and L206 (O——O). Results are expressed as percentage binding as a function of total Ig concentration (g/l). The labeled antigen was R α1-m (A) or H α1-m (B).

Radiolabeled R α1-m was precipitated in the PEG test by antiserum L 206 and high concentrations of the monoclonal anti-human α1-m antibody H23 (fig. 4), but not by rabbit anti-human α1-m (L 177). Conversely, H α1-m was precipitated by L 177 and H23 as well as by L 206. Cross-reactivity between Hα1-m and Rα1-m was further documented by competitive studies shown in table 1. Three labeled α1-m preparations were used : the above described Rα1-m, H α1-m purified as described by Vincent et al. (11), and H α1-m immunopurified with L 206.

Table I. Inhibition of binding of human or rat α1-m, by 1 µg/ml of purified H α1-m and R α1-m.

| Labeled antigen | H | | H+ | | | | R | |
|---|---|---|---|---|---|---|---|---|
| Antibody | H 23 | L 206 | H 23 | H 23 | L 206 | L 206 | H 23 | L 206 |
| (mg/ml) | (1) | (2.5) | (1) | (0.05) | (2.5) | (0.1) | (1) | (2.5) |
| Buffer | 60 | 77.9 | 100 | 61.3 | 91.8 | 29 | 34.5 | 30 |
| H α1-m | 6.8 | 27.3 | 94 | 36.4 | 11.3 | 8 | 33.4 | 20.4 |
| R α1-m | 40.8 | 60.7 | 92 | 45.5 | 88.6 | 28.5 | 34.9 | 26 |
| Control | 5 | 5 | 3.4 | 4 | 3.4 | 4 | 18.6 | 18.6 |

+ Human α1-m immunopurified on L 206 immunosorbent after labeling. The amount of labeled antigen introduced in the test was 1:20 of the amount introduced in series H or R.

H α1-m was precipitated by H 23 and L 206 and this precipitation was inhibited to a greater extent by the human antigen than by the rat homologue. Immunopurified labeled H α1-m was totally precipitated by both H 23 and L 206. However only the precipitation by L 206 could be inhibited. The absence of inhibition of H 23 precipitation is probably due to a great antibody excess because an inhibition can be observed if a lower concentration of H 23 is used. Furthermore, the labeled R α1-m was precipitated, but to a lesser degree. The binding of R α1-m by L 206 was slightly inhibited by purified H α1-m.

**Distribution of α1-m in rat serum fractions.** One ml of rat or human serum was chromatographed on S 200 gel. Each fraction between tubes 10 and 26 was concentrated to a volume of 0.2 ml and essayed for α1-m activity using either labeled H α1-m and H 23 or labeled R α1-m and L 206. In both cases similar profiles of activity were obtained : part of the activity was recovered near the excluded volume, and a part as a second peak in the 30 000 d region with a shoulder in a higher MW region for rat serum (fig. 5).

## DISCUSSION

The R α1-m described in this report is similar to H α1-m for a number of criteria. Both proteins migrate in identical positions on SDS-PAGE showing a

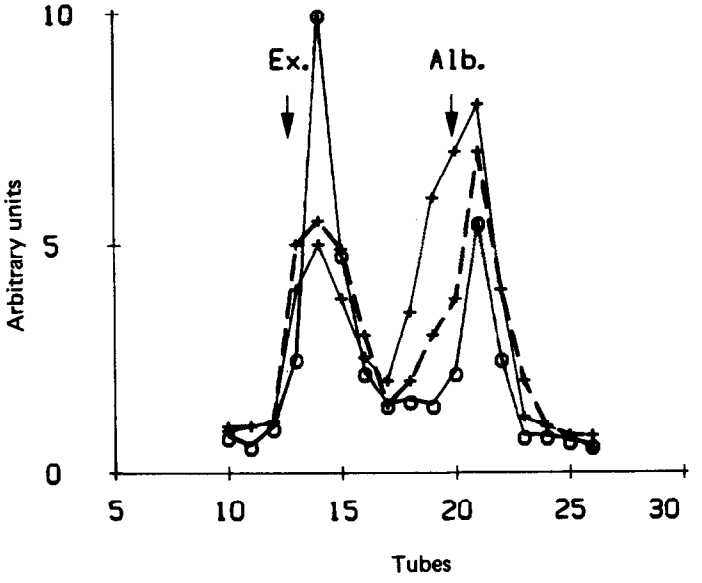


Figure 5 Chromatography of normal human serum ( O ) or normal rat serum ( + ) on Sephacryl S200 gel. The eluted fractions were assayed for competition in a radioimmunoassay either in a system with human labeled α1-m and H23 antibodies (——), or in a system with rat labeled α1-m and L206 antiserum (— —). Results were calculated using normal serum of the corresponding species as standard, the inhibition given by serum diluted 1:4 is refered as 10 arbitrary units.

homogenous band corresponding to an apparent molecular weight of 30,000d. Both are associated with the brown pigment and can be adsorbed on Con A. R α1-m is heterogenous in charge showing at least 5 distinct bands in chromatofocussing in the pH range of 4.51-4.98 ; whereas H α1-m shows the same profile although the pI values are slightly lower: 4.3-4.9 (11). In agarose gel electophoresis at pH 8.8, R α1-m migrates in the fast α1 zone, as does H α1-m. Fluorescence studies were done in comparison with two different preparations of H α1-m (data not shown). The fluorescence excitation spectra of R α1-m and H α1-m show the same three peaks, although for H α1-m the peaks at 357 and 380 had comparable intensity. The emission spectra were comparable with both R and H α1-m. Most of R α1-m properties are shared by guinea-pig α1-m (8) which, however, differs from R α1-m by a slightly lower MW, lower pI values and slightly faster electrophoretic mobility in agarose gel at pH 8.8. Also, the absorption spectrum of R α1-m was identical to that of guinea pig α1-m (8). The fluorescence excitation spectrum of R α1-m showed three peaks at 290, 357 and 380. Only two of these were observed with guinea pig α1-m. The emission spectrum with the excitation maintained at 380 nm showed a broad peak in the 475 nm region as that of guinea pig. The additionnal peak detected at 330 nm with R α1-m and H α1-m can be observed only with an excitation wavelength below 310 nm, (a condition which had not been studied by Akerström) (8).

The formation of stable dimers has been reported for H α1-m (3) and guinea-pig α1-m (8). R α1-m appeared to be even more prone to dimer formation as most of the protein being recovered as a 60,000d band in non reducing SDS-PAGE and as a 30,000d band after reduction. Moreover spontaneous dimer or polymer formation is indicated by the high non specific precipitation in 8 % PEG solution.

The immunological cross-reaction between H α1-m and R α1-m shows that both proteins share at least one epitope. This common antigenic determinant was recognized by anti-human α1-m monoclonal antibody H 23, and quite likely, as well, by the rabbit anti-rat α1-m serum, L 206. The low binding of R α1-m by H23 and L 206 suggests that the detected epitope was masked by R α1-m aggregation. This cross-reactivity was confirmed by immunohistochemical studies on various rat tissues showing an identical pattern of staining with these two reagents (Bouic et al., submitted for publication) as well as the profile of α 1-m activity after chromatography of rat serum.

## REFERENCES

1. Ekström, B., Peterson, P.A., and Berggard, I. (1975) Biochim. Biophys. Res. Comm. 65, 1427-1433.
2. Tejler, L., and Grubb, A.O. (1976) Biochim. Biophys. Acta 439, 82-94.
3. Ekström, B., and Berggard, I. (1977) J. Biol. Chem. 252, 8048-8057.
4. Svensson, L., and Ravnskov, U. (1976) Clin. Chim. Acta 73, 415-422.

5. Seon, B.K., and Pressman, D. (1978) Biochemistry17, 2815.
6. Tagaki, K., Kin, K., Itoh, Y., Kawai, T., Kasahara, T., himoda, T., and Shikata, T. (1979) J. Clin. Invest. 63, 318-35.
7. Berggard, B., Ekström, B., and Akerström, B. (1980) Scand. J. Clin. Lab. Invest. K40, suppl. 154, 63-71.
8. Akerström, B., and Berggard, I. (1979) Eur. J. Biochem. 101, 215-223.
9. Evans A.P., and Dail W.G. (1974) Lab. Invest. 30, 704-715.
10. Bouic, P., Vincent, C., Revillard, J.P., and Brochier, J. (1981) in Physiological Peptides and New Trends in Radioimmunology (Ch.A. Bizollon ed.) pp 307-313, Elsevier/North-Holland Amsterdam.
11. Vincent, C., Revillard, J.P., Bouic, P., and Brochier J. (1981) in Protides of the Biological Fluids (H. Peeters ed.) pp 777-780, Pergamon Press Ltd Oxford.
12. Hunter, W.M. (1978) in Handbook of Experimental Immunology (D.M. Weir, ed.), vol. 1, pp 14.1-14.40, Blackwell Scientific Oxford.
13. Vincent, C., and Revillard, J.P. (1980) Mol. Immunol. 17, 723-728.
14. Jeppson, J.O., Laurell, C.B., and Frauzen, B. (1979) Clin. Chem. 25, 629-638.
15. Laemmli, U.K. (1970) Nature 227, 680-685.