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CHROMATOGRAPHIC METHODS FOR PURIFICATION OF LEUKOCYTE INTERFERON

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SUMMARY

A chromatographic method has been developed for obtaining interferon products of three types: (a) with a specific activity of 10^5 I.U./mg of protein (on the basis of leukocyte interferon); (b) with a specific activity of 10^6 I.U./mg of protein (on the basis of leukocyte interferon); (c) a near-homogeneous preparation with an activity of $2 \cdot 10^8$–$4 \cdot 10^8$ I.U./mg of protein (for leukocyte and recombinant interferons). In the first two cases, only gel permeation chromatography (GPC) is used, which yields interferon preparations containing the oligomeric forms of interferon and also such biologically active proteins as factor D and interleukin-1. In the last case, GPC and immunoaffinity chromatography may be conveniently supplemented by interferon purification by ion exchange to remove immunoglobulins and the products formed by their hydrolytic or proteolytic decomposition.

INTRODUCTION

Discovered by Isaacs and Lindemann[1], interferon has become —as a result of fundamental investigations on the interaction of viruses with cells— an object for research and development in biotechnology on an unprecedented scale. Many scientific groups have devoted their efforts to the study of the properties of interferon and its potential for application as an antiviral and antitumoral drug, involving a high level of resources. The forecasts of different firms and research centres show this expenditure to be quite justified, as the expected requirements for interferon drugs are estimated to be worth up to $\$10^9$ by 1990[2].

The production of interferon involves three basic stages: (a) cultivation of interferon-producing cells (human leukocytes, and also cell strains obtained by genetic engineering; (b) isolation and purification of the interferon to a predetermined degree of homogeneity and (c) preparation of interferon in its end-use form, suitable for subsequent clinical administration. The fact that none of these production stages has been developed to a sufficient extent prevents the complete characterization of interferon preparations. One of the most important recent achievements is the development of chromatographic methods for the isolation and purification of interferon preparations which permit them to be obtained in a practically homogeneous state.

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The present work deals with the problems that arise at the chromatographic stages of obtaining interferon preparations.

EXPERIMENTAL

Human leukocyte interferon was obtained as described previously[3]. Bacterial recombinant interferon, an experimental species, was donated by the All-Union Research Institute of Genetics and Selection of Industrial Microorganisms. Screening for interferon activity was based on suppression of the cytopathic effect of vesicular stomatitis virus (VSV), strain Indiana[3]. The interferon activity was also determined by radioimmunoassay and enzyme immunoassay[4]. The contents of factor D and interleukin-1 in the interferon preparations were determined according to methods described previously[3,5].

The family of hybridomas, producing monoclonal antibodies (MAB) to α_2-interferon, and methods for their purification, are given in ref. 4.

The sorbents for immunoaffinity purification of interferon were obtained by condensation of MAB LBP5A6 on Sepharose 4BCL by the hydrazide method, and permitted protein concentrations on the sorbent of 3 mg/ml of swollen gel. The sorbent capacity for interferon increased linearly with increasing concentration of the immobilized protein and reached $2.08 \cdot 10^8$ I.U./ml of swollen gel. For gel permeation chromatography (GPC) the sorbents Ultrogel AcA-54, Sephacryl S-200 (Pharmacia), TSK 2000SW and TSK HW-50 (Toyo Soda); for high-performance liquid chromatography (HPLC), Spherisorb 5 ODS (Phase Separations), and —at the stage of ion-exchange purification of interferon to remove admixtures of MAB and their hydrolytic fragments— the carboxy cation exchangers Soloza K, synthesized in this Institute[6,7], were employed.

Electrophoretic assays were carried out as described previously[3].

The content of MAB or their fragments in interferon preparations was determined by enzyme immunoassay with rabbit anti-mouse antibodies[8].

RESULTS

The general schemes used for the production of leukocyte and bacterial interferon preparations, as developed by this Institute (in the last case in cooperation with the All-Union Research Institute of Genetics and Selection of Industrial Microorganisms, Main Board for Microbiological Industry), are shown in the Figs. 1 and 2.

For leukocyte interferon these schemes involve stepped salt precipitation, GPC in the presence of a disaggregating agent (8 *M* urea), immunoaffinity chromatography (IAC) and ion-exchange chromatography (IEC) on carboxy polyelectrolytes, Soloza K. For recombinant bacterial interferon, the purification comprised IAC between the stages of sorption-desorption on the ion-exchange polyelectrolytes, Soloza A and K. In both cases the preparations obtained had a specific activity of $2 \cdot 10^8$ I.U./mg, comparable with that for homogeneous preparations[9].

The use of GPC, particularly high-performance GPC (on TSK 2000SW), for purification of interferons showed the presence of oligomeric forms of interferon, mainly dimers, in the recombinant interferon preparations. The data presented in Fig. 3 show that the oligomers result from the formation of covalent bonds involving

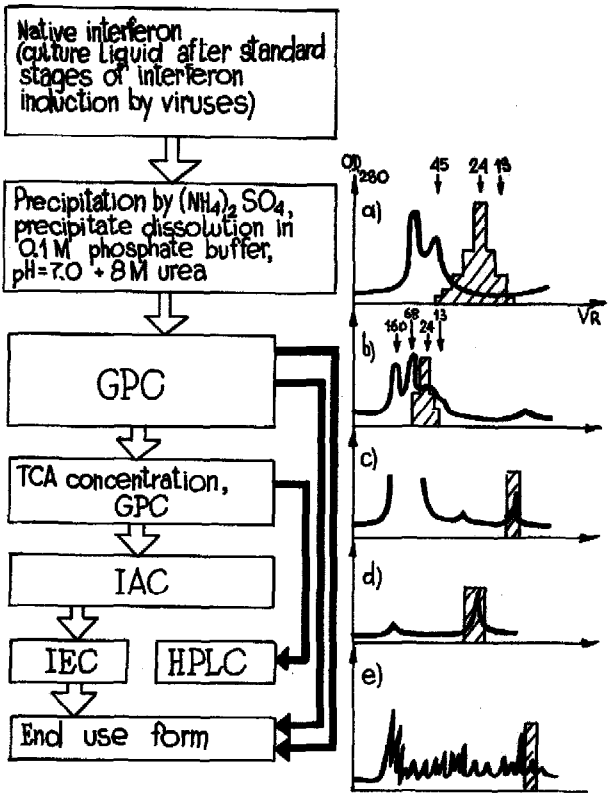

Fig. 1. General flow diagram for the preparation of human leukocyte interferon. (a) GPC, Ultrogel AcA-54, K100/100 column, 0.1 *M* phosphate buffer, pH 7.0, flow-rate 400 ml/h; specific activity: 10^5 I.U./mg; yield, 100%. (b) GPC, trichloroacetic acid (TCA) concentrate on Sephacryl S-200, K50/100 column, buffer as in (a), flow-rate 100 ml/h; specific activity: $2 \cdot 10^6$ I.U./mg; yield, 80%. (c) IAC, LBP5A6-Sepharose 4BCL, K9/15 column, flow-rate 40 ml/h, sorption in 0.1 *M* phosphate buffer, pH 7.0, washing with same buffer, but containing 0.5 *M* sodium chloride, elution with 0.1 *M* glycine buffer, pH 3.0, containing 0.5 *M* sodium chloride; specific activity: $2 \cdot 10^8$ I.U./mg; yield, 80%. (d) IEC, Soloza K8/24, K9/15 column, flow-rate 7 ml/h, 0.05 *M* Tris–HCl buffer, pH 5.0, sorption in glycine buffer, see (c), adjustment to pH 5.0 with 0.01 *M* sodium hydroxide, gradient elution with 0.1 *M* Tris–HCl, pH 9.5, up to pH 9.5; specific activity: $>2 \cdot 10^8$ I.U./mg; yield, 100%. (e) HPLC, Spherisorb 5 ODS, 250 mm × 10 mm, flow-rate 2 ml/min, continuous sorption by pump, washing with 1 *M* acetate buffer, pH 4.0, elution with a multilinear isopropanol gradient to a final concentration of 40%; specific activity: $2 \cdot 10^8$ I.U./mg; yield, 60%. Values at the arrows indicate the molecular weight (in kilodaltons) of the fractions.

disulphide bridges rather than from intermolecular association. Morchend *et al.*[10] had previously come to the same conclusion while studying the properties of interferon by electrophoresis in polyacrylamide gel (PAG) under reducing and denaturing conditions. Whether such forms exist in the case of leukocyte interferon has not been possible to establish due to the rather low interferon concentration in solution and the insufficient accuracy of the interferon titration method based on suppression of the cytopathic effect of viruses. However, there is the possibility of the existence of such forms of interferon, and this poses the problem of variations in their biological effects.

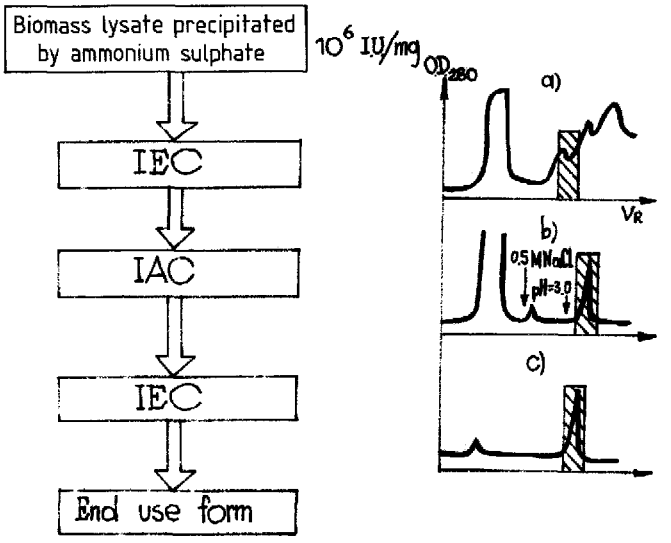

Fig. 2. General flow diagram for preparation of bacterial α-interferon. (a) IEC, $5 \cdot 10^7$ I.U./mg; yield, 80%, Soloza A40/60, K50/30 column, 0.05 *M* Tris–HCl buffer, pH 7.2, flow-rate 50 ml/h, elution with a linear gradient to 0.5 *M* sodium chloride in the same buffer, interferon elution zone 0.15–0.3 *M* sodium chloride. (b) IAC, $2 \cdot 10^8$ I.U./mg; yield, 90%, LBP5A6-Sepharose 4BCL, K50/30 column, sorbent layer 100 mm, sorption in same buffer as in (a) but containing 0.2 *M* sodium chloride, washing with 0.02 *M* phosphate buffer containing 0.5 *M* sodium chloride, elution with 0.1 *M* glycine buffer, pH 3.0, containing 0.5 M sodium chloride, flow-rate 200 ml/h. (c) IEC, $2 \cdot 10^8$ I.U./mg; yield, 90%, conditions as in Fig. 1d.

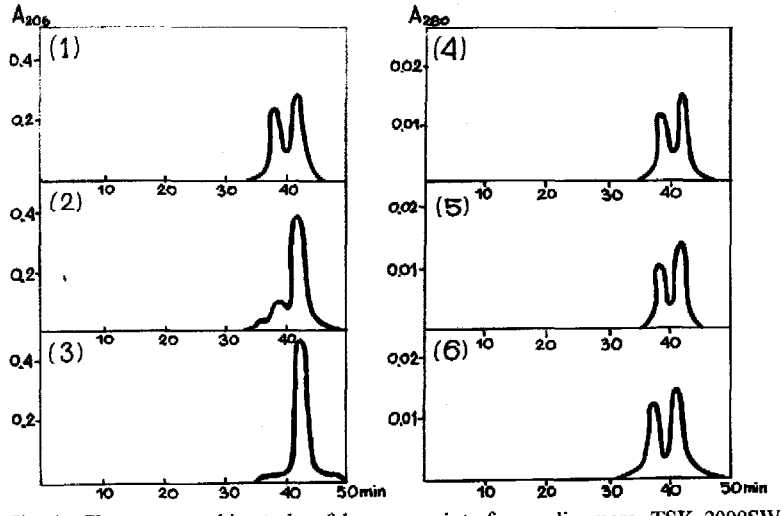

Fig. 3. Chromatographic study of human α_2-interferon oligomers. TSK 2000SW 300 mm × 7.5 mm column, 0.1 *M* phosphate buffer, pH 7.0, 20°C, flow-rate 0.5 ml/min. (1) Starting material; (2) incubation for 24 h in 5% 2-mercaptoethanol; (3) incubation for 72h in 5% 2-mercaptoethanol; (4) incubation for 24 h in 1% SDS; (5) incubation for 24 h in 8 *M* urea; (6) starting material, separation in acetate buffer, pH 4.0.

Another feature of GPC in interferon purification is that such biologically active proteins as interleukin-1 and factor D of the complement system, both having molecular weights similar to that of interferon (Fig. 4), appear the α-interferon zone, in spite of the high degree of purification with reference to other proteins (up to 100 times). Both proteins can promote antiviral and antibacterial activities in human α-interferon obtained as a result of biosynthesis, salt precipitation and GPC.

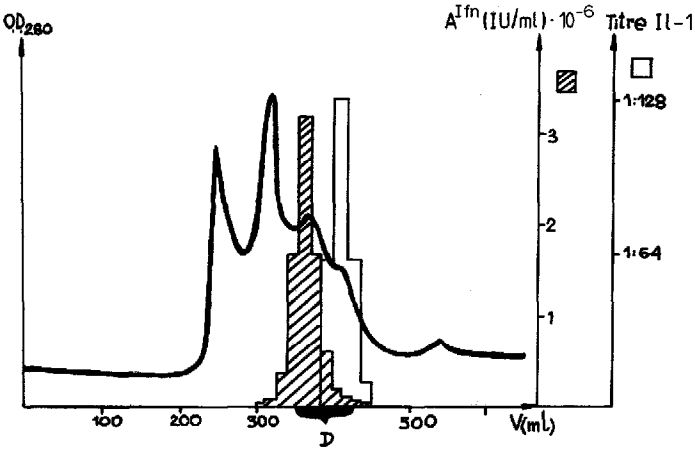

Fig. 4. Preparative isolation of leukocyte interferon. TSK HW-50, K50/100 column, flow-rate 100 ml/h, 0.1 *M* phosphate buffer, pH 7.0. Separation of TCA concentrate (40 ml) obtained from 3000 ml of partially refined product (see Fig. 1a). Marked are the elution zones of interferon, interleukin-1 and factor D of the complement system. Interferon activity (A^{Ifn}, IU/ml) is defined in international units (IU) in terms of standard preparation MRC B69/19. The titre of interleucin 1 (IL-1) is defined as the last two-fold dilution leading to 50% of maximum count per minute increase in mouse thymocyte costimulation assay[17].

Interleukin-1 is known to stimulate B-lymphocyte proliferation, *i.e.*, to stimulate the immune response to the antigen introduced. In interferon preparation, interleukin-1 is normally detectable in 1:32 to 1:64 titres. Factor D is contained in interferon preparations at a concentration of *ca.* 20 ng per 10^4 I.U. of interferon, *i.e.*, $2 \cdot 10^{-5}$ mg D/mg of protein.

The antibacterial effect of the factor D has been demonstrated both by *in vitro* experiments on the increase in lytic activity of the complement against bacterial cells and by *in vivo* experiments carried out on a keratoconjunctivitis model induced by a virulent *Escherichia coli* strain in guinea pigs. In the latter case, an intravenous injection of homogeneous factor D (50 μg) helped to reduce the infection period 1.5-fold.

This shows that leukocyte α-interferon with a specific activity of 10^5 I.U./mg of protein, *i.e.*, after GPC, may be promising for local administration.

A higher degree of purification of α-interferon preparations can be achieved by IAC using immobilized monoclonal α-interferon antibodies or by high-performance hydrophobic interaction liquid chromatography (Fig. 5).

To obtain high-purity human α-interferon (natural species or recombinant α_2), I utilized IAC on MAB LBP5A6 (Fig. 6) immobilized on Sepharose 4BCL by the

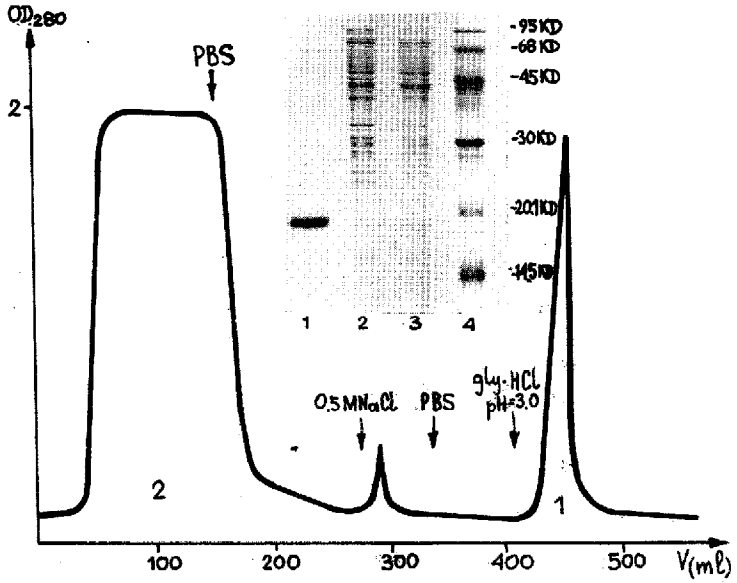

Fig. 5. Immunoaffinity purification of α_2-interferon on MAB LBP5A6 immobilized on Sepharose 4BCL, C_{MAB} = 3 mg/ml. Column 100 mm × 50 mm (see Fig. 2b for conditions). Electrophoresis in 15% PAG: 1 = purified α_2-interferon; 2 = non-sorbed material; 3 = starting product; 4 = molecular weight markers. PBS = phosphate buffered saline; KD = kilodalton.

hydrazide method in a concentration of 3 mg/ml (Fig. 6). Despite the apparent simplicity of the above method which yields almost homogeneous target protein in a single stage from greatly diluted mixtures highly contaminated with foreign proteins, the method has some limitations.

Thus the conditions used for elution are normally rather severe. They may involve either low pH (the standard value is 2.0), concentrated thiocyanate solutions (1–3 *M*) or solutions of disaggregating components (urea; guanidine chloride; sodium dodecyl sulphate, SDS), which may result in matrix hydrolysis and ligand fragmentation. Also, the agent used to coat the immunoaffinity column usually contains

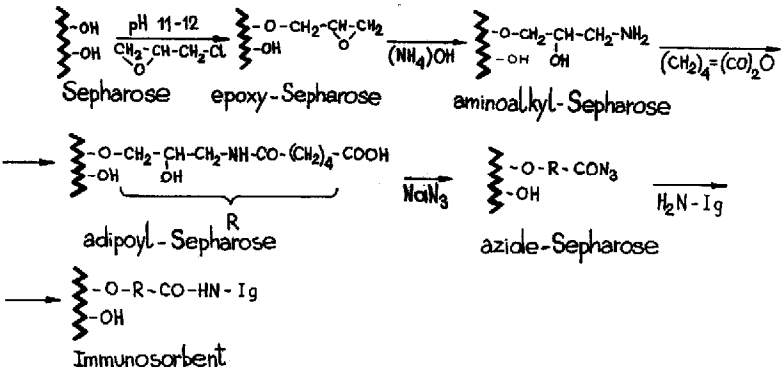

Fig. 6. Flow diagram for preparation of immunosorbents.

proteases, which also favour MAB decomposition. MAB determination in interferon preparations after IAC by using immunometric methods (dot-blotting in radioimmuno- and immunoenzymatic versions and solid-phase immunometry) gave the MAB (or MAB fragments) content in α-interferon preparations as 1% of the total proteins. Considering that the therapeutic α-interferon dose is 0.01 mg, the amount of a foreign protein administered to the patient should not exceed 100 ng. However, even this amount should be removed.

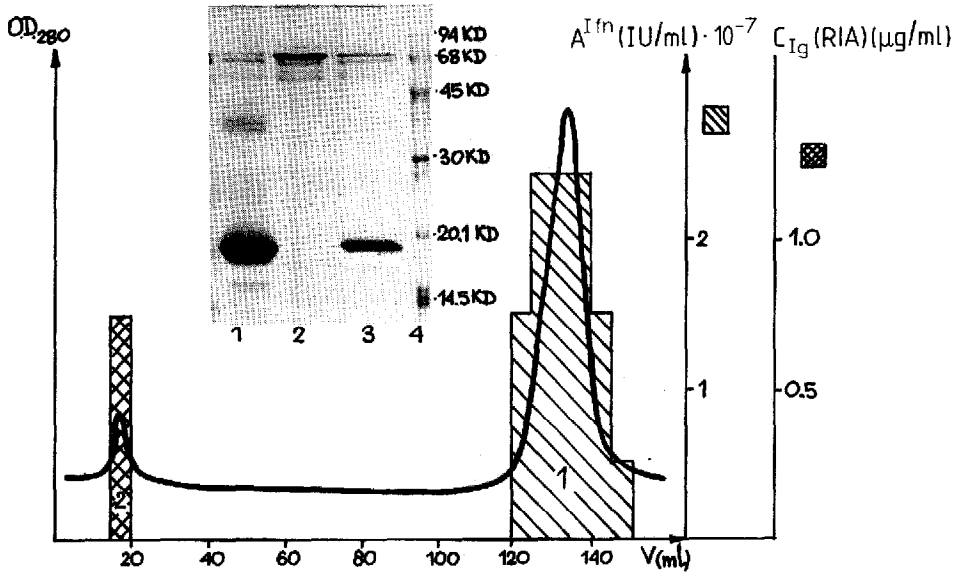

Fig. 7. Ion-exchange purification of α_2-interferon, Soloza K8/24, column K9/15 (see Fig. 2c (for conditions). Electrophoresis in 15% PAG: 1 = purified product; 2 = non-sorbed material; 3 = starting material; 4 = molecular weight markers. C_{Ig}(RIA) (μg/ml) is the immunoglobulin (or its fragments) concentration defined by radioimmunoassay.

IEC using carboxy cation exchangers may be a suitable method for separating MAB and interferon. Considering the similar values of the isoelectric points of LBP5A6 and α_2-IF, the poorly controlled p*I* values of LBP5A6 fragments and the high hydrophobicity of α_2-IF, it has been deemed convenient to use hydrophobic IEC on Soloza carboxy cation exchangers. These latter compounds are copolymers of methacrylic acid, butyl methacrylate and methylenedimethacrylamide with various ratios of the first two comonomers. By proper selection of copolymerization conditions, the Soloza K polyelectrolytes can also be in the form of highly permeable sorbents, suitable for separation of proteins with molecular weights of up to 10^5. The main physico-chemical properties of these sorbents are summarized in Table I. Fig. 7 presents data for the selection of conditions for the separation of MAB and interferon. Although the extent of purification by protein removal is rather insignificant at this stage, it permits the removal of residual immunoglobulins and the products of hydrolytic or proteolytic decomposition of immunoglobulin–polysaccharide sorbent complexes.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF SOLOZA K CATION EXCHANGERS OF VARIOUS COMPOSITIONS[6]

E_{BSA} = maximum capacity toward bovine serum albumin at pH 5.0 with 0.1 *M* phosphate buffer at room temperature. $\bar{D}_s$ = surface averaged mean pore diameter of the sorbent.

*Ratio of BMA:MAA:MDAA**	*Specific surface area of pore volume*[16] *(m^2/cm^3)*	$\bar{D}_s$[16]	E_{BSA}[6] *(mequiv. g dry sorb.)*	$\Delta\Phi^0$ *for hexanol sorbtion (kJ/mg)*
0:60:40	180	230	–	0.6
10:50:40	190	210	1000	1.4
20:40:40	200	200	930	2.2
30:30:40	150	260	800	3.8
40:20:40	120	340	800	4.5
60:10:40	120	320	–	4.2

* BMA, MAA, MDAA = butylmethacrylate, methacrylic acid and methylenediacrylamide (cross-linking agent).

DISCUSSION

The chromatographic methods of purification of α-interferon have been developed by taking into account such properties of interferon as its size ($\sqrt{r^2} < 2.0$ nm), isoelectric points (p*I* ≈ 5.8–7.2), stability over a wide range of pH (2.0 < pH < 10 for α_2-interferon) and high hydrophobicity (≈35% of hydrophobic amino acid residues), and involve GPC, IEC, IAC and reversed-phase chromatography in various combinations. Also considered were the data obtained by Levy *et al.*[11] and Shively *et al.*[12] which show that the α-interferon molecule is composed of 165 amino acid residues in the sequence shown in Fig. 8.

The possible secondary and tertiary structures in the interferon molecule, *i.e.*, the dextrorotatory group of four α-helical segments as proposed by Stenberg and Cohen[13] and the five α-helical segments group proposed by Zav'yalov[14,15], show that there are a large number of hydrophobic residues on the external surface of the molecule, which determine the high hydrophobicity of the molecule as a whole. The latter factor may given rise to the formation of interferon oligomers (mainly dimers) detectable by high-performance GPC.

The hydrophobic properties of interferon were also taken in account in selecting the conditions (for separation of interferon and MAB). The similarity of the p*I* values of these proteins precludes the application of hydrophilic carboxy sorbents of the CM-Sepharose type. In the case of the Soloza K ion exchangers the order of elution is determined both by the hydrophobic properties of the protein and by the properties of the sorbent.

It was also possible to develop a method of separating interferon and ballast proteins by means of HPLC on reversed-phase sorbents (particularly, Spherisorb 5 ODS). The method was used as the last stage to accumulate pure natural α-interferon

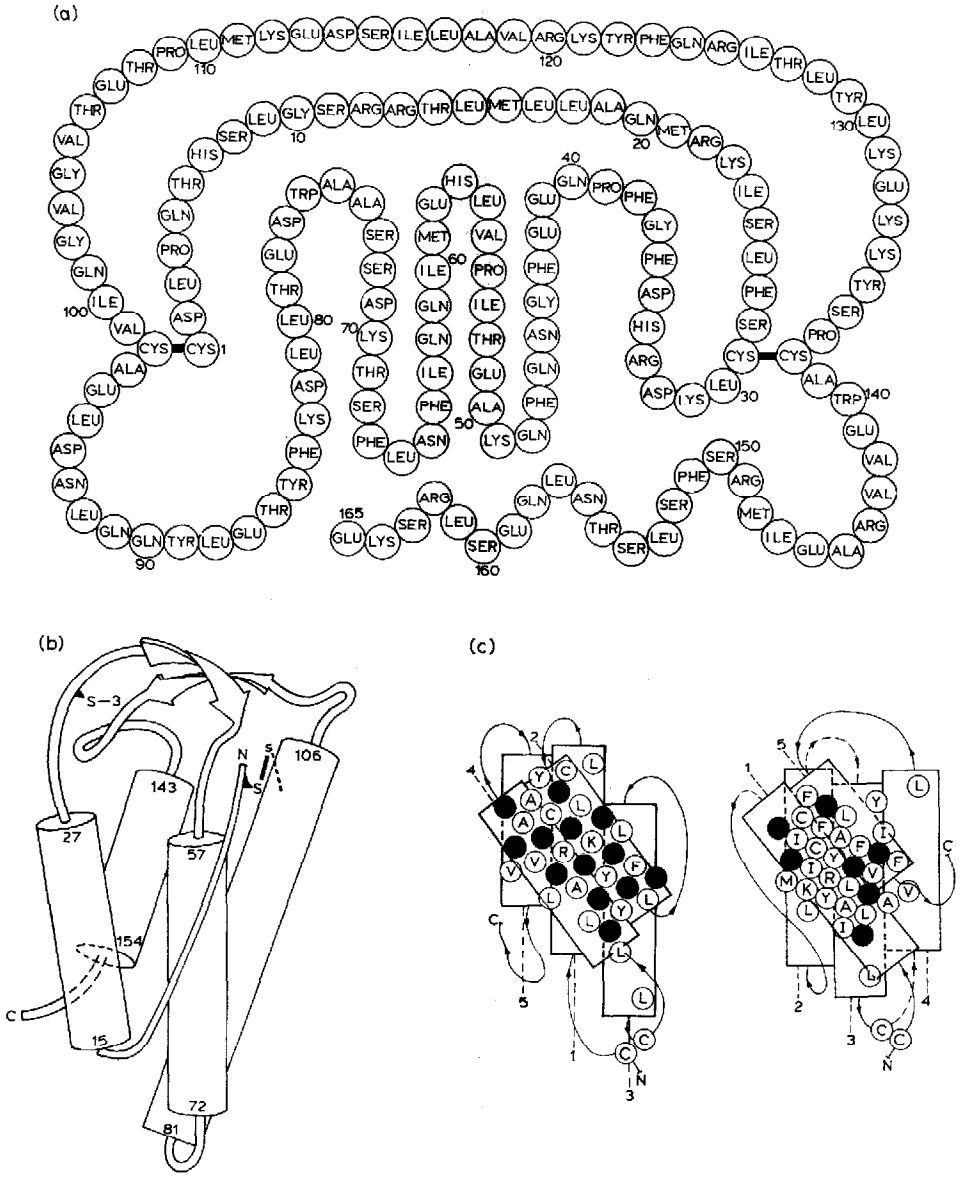

Fig. 8. Structure of human α-interferon. (a) Primary structure[11,12]; (b) spatial structure[13]; (c) spatial structure[14,15] (filled circle represent hydrophobic amino acids). 1–5 are the numbers of α-helical segments. One letter codes for amino acids are used.

for the preparation of hybridomas producing MAB. The preparation with a specific activity of $1 \cdot 10^6$–$5 \cdot 10^6$ I.U./mg ($2 \cdot 10^7$–$10 \cdot 10^7$ I.U. in 6 *M* urea) was subjected to continuous sorption on 250 × 10 mm Spherisorb 5 ODS columns, and eluted with a multilinear gradient of isopropanol in a 1 *M* acetate buffer, pH 4.0. Optical methods were used to detect the peak corresponding to the biological activity of

interferon. The degree of purification achieved at this stage was *ca.* 100, with a specific activity equal to $2 \cdot 10^8$ I.U./mg, which corresponds to that of homogeneous α-interferon. This mode of purification is thought convenient for obtaining small quantities of highly pure preparations without separation into subclasses, even though it is inferior in productivity to MAB immunoaffinity chromatography.

Thus, the stages of non-specific purification of α-interferon must be based on a consideration of the two main properties of this molecule: low molecular weight and high hydrophobicity. In combination with a specific method such IAC, this allows almost homogeneous interferon preparations to be obtained and enables the characteristics of both types of interferons to be studied and compared.

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