

Сколько нужно чистого белка для:

- масс спектрометрии
- определения аминокислотной последовательности
- Электрофоретический анализ
- Определение ферментативной активности
- Физико-химические методы исследования (ЯМР и др.)
- Препараты для исследований
- Диагностические и терапевтические препараты
- Препараты плазмы крови, кровезаменители
- 10 – 100 пг
- 100 пг – 100 нг
- 10 нг – 10 мкг
- 1 – 10 мкг
- 1 – 100 мг
- 1 мг – 10 грамм
- От 1 кг до 10000 кг
- Тоннажное производство

Наиболее объемные производства белков для фармакологии

Table 1. Very Large Scale cGMP Purification Processes

product	est annual prod (tons)	major producers	source	purification process	sale price (\$/g)	licensure date
HSA	500	Talecris CSL Behring Baxter	plasma	Cohn fractionation ultrafiltration	3	1940s
IgIV	80 ^a	Talecris CSL Behring Baxter	plasma	Cohn fractionation ultrafiltration	60–70	1980s
insulin	10	Eli Lilly Novo Nordisk Sanofi Aventis	microbial	centrifugation + disruption ^d 6 columns (SEC + RP) crystallization	500	1982
Rituxan	1	Genentech Lonza ^b	CHO	centrifugation 3 columns 2 ultrafiltration steps ^e	4,000	1987
Enbrel	1	Amgen Wyeth BI ^c	CHO	microfiltration 3 columns 4 ultrafiltration steps ^f	4,000	1998
10-ton Mab	10	hypothetical	CHO	centrifugation 2 columns 2 ultrafiltration steps	hypothetical	

^a Based on ref 30. ^b Produced under contract. ^c Boehringer Ingelheim. ^d Based on the Lilly process described in ref 8. ^e Based on ref 31. ^f Based on ref 32.

Table 2. Details of 10-Ton mAb Purification Process Unit Operations

Centrifugation and Harvest	
centrifuge flowrate	3,750 L/h
pad filter volumetric challenge	200 L/m ²
0.2 μm Prefilters	
volumetric challenge (varies with stream)	100–1000 L/m ²
Protein A Chromatography	
column diameter	200 cm
bed height	50 cm
bed volume	1,500 L
[mAb] in load	5 g/L
linear velocity	300 cm/h
dynamic binding capacity	50 g/L
elution volume	2.5 CVs (3,750 L)
Anion-Exchange Chromatography	
column diameter	120 cm
bed height	25 cm
bed volume	320 L
[mAb] in load	20 g/L
linear velocity	150 cm/h
column load capacity ^a	250 g/L
pool volume	4,710 L
Virus-Retaining Filtration	
membrane area	8 m ²
average flux	40 L/m ² h
[mAb] in load	20 g/L
Ultrafiltration/Diafiltration	
[mAb] in load	18–19 g/L
membrane area	100 m ²
average flux	30 L/m ² h
[mAb] in load	18 g/L
diafiltration volume	10 diavolumes
target mAb concentration	100 g/L

^a The AEX step is defined not by the product's dynamic binding capacity, as in the ProA step, but the total product loaded; only a fraction of the product binds under weak partitioning conditions.

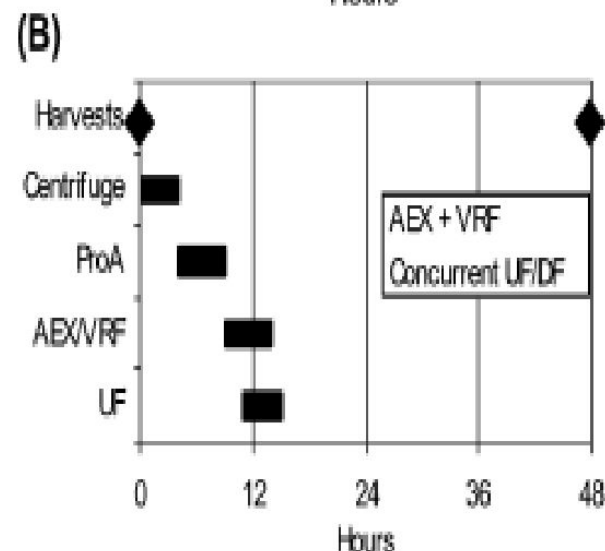
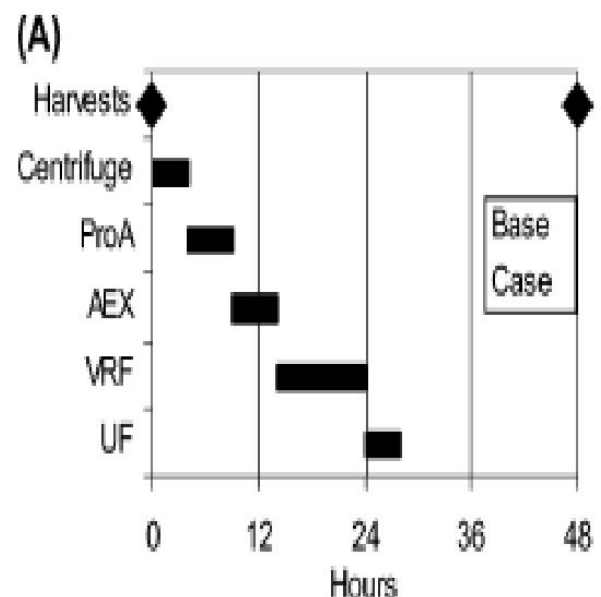


Figure 4. Production schedule for the purification operations: (A) base case, (B) combination of the AEX and VRF steps along with concurrent UF/DF.

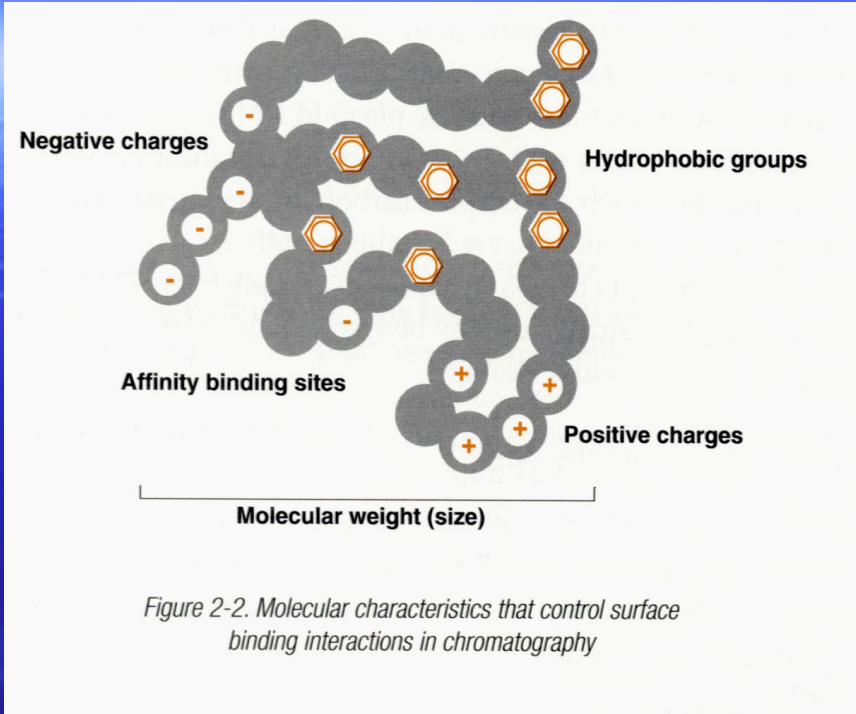
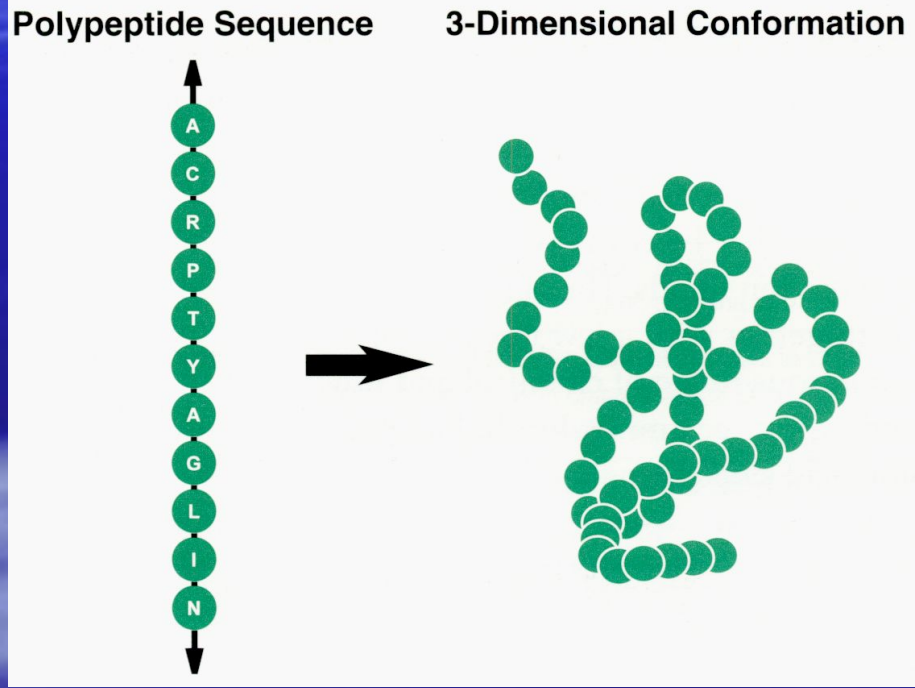
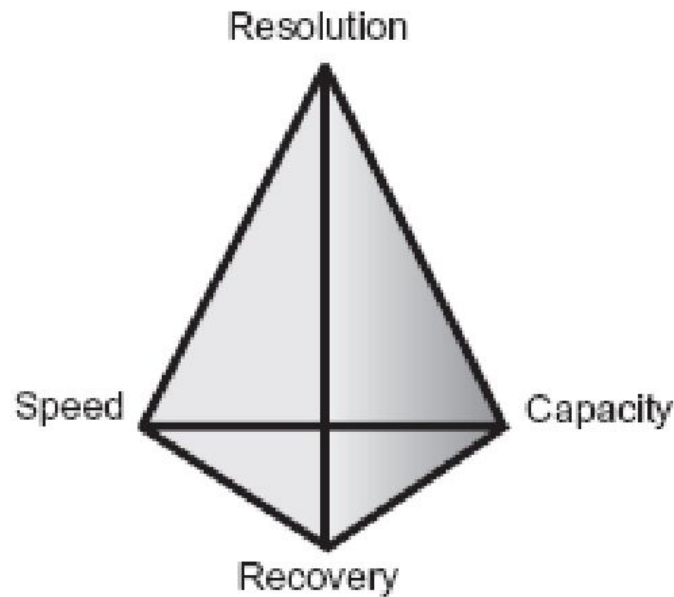


Figure 2-2. Molecular characteristics that control surface binding interactions in chromatography

Selection and Combination of Purification Techniques



Every technique offers a balance between resolution, capacity, speed and recovery.

Minimise sample handling
Minimise number of steps
Use different techniques at each step

Protein property	Technique
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, ligand specificity or hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

Define objectives

for purity, activity and quantity required of final product to avoid over or under developing a method

Define properties of target protein and critical impurities

to simplify technique selection and optimisation

Develop analytical assays

for fast detection of protein activity/recovery and critical contaminants

Minimise sample handling at every stage

to avoid lengthy procedures which risk losing activity/reducing recovery

Minimise use of additives

additives may need to be removed in an extra purification step or may interfere with activity assays

Remove damaging contaminants early

for example, proteases

Use a different technique at each step

to take advantage of sample characteristics which can be used for separation (size, charge, hydrophobicity, ligand specificity)

Minimise number of steps

extra steps reduce yield and increase time, combine steps logically

Table 1. Protein properties and their effect on development of purification strategies.

Sample and target protein properties	Influence on purification strategy
Temperature stability	Need to work rapidly at lowered temperature
pH stability	Selection of buffers for extraction and purification Selection of conditions for ion exchange, affinity or reversed phase chromatography
Organic solvents stability	Selection of conditions for reversed phase chromatography
Detergent requirement	Consider effects on chromatographic steps and the need for detergent removal. Consider choice of detergent.
Salt (ionic strength)	Selection of conditions for precipitation techniques, ion exchange and hydrophobic interaction chromatography
Co-factors for stability or activity	Selection of additives, pH, salts, buffers
Protease sensitivity	Need for fast removal of proteases or addition of inhibitors
Sensitivity to metal ions	Need to add EDTA or EGTA to buffers
Redox sensitivity	Need to add reducing agents
Molecular weight	Selection of gel filtration media
Charge properties	Selection of ion exchange conditions
Biospecific affinity	Selection of ligand for affinity medium
Post translational modifications	Selection of group-specific affinity medium
Hydrophobicity	Selection of medium for hydrophobic interaction chromatography

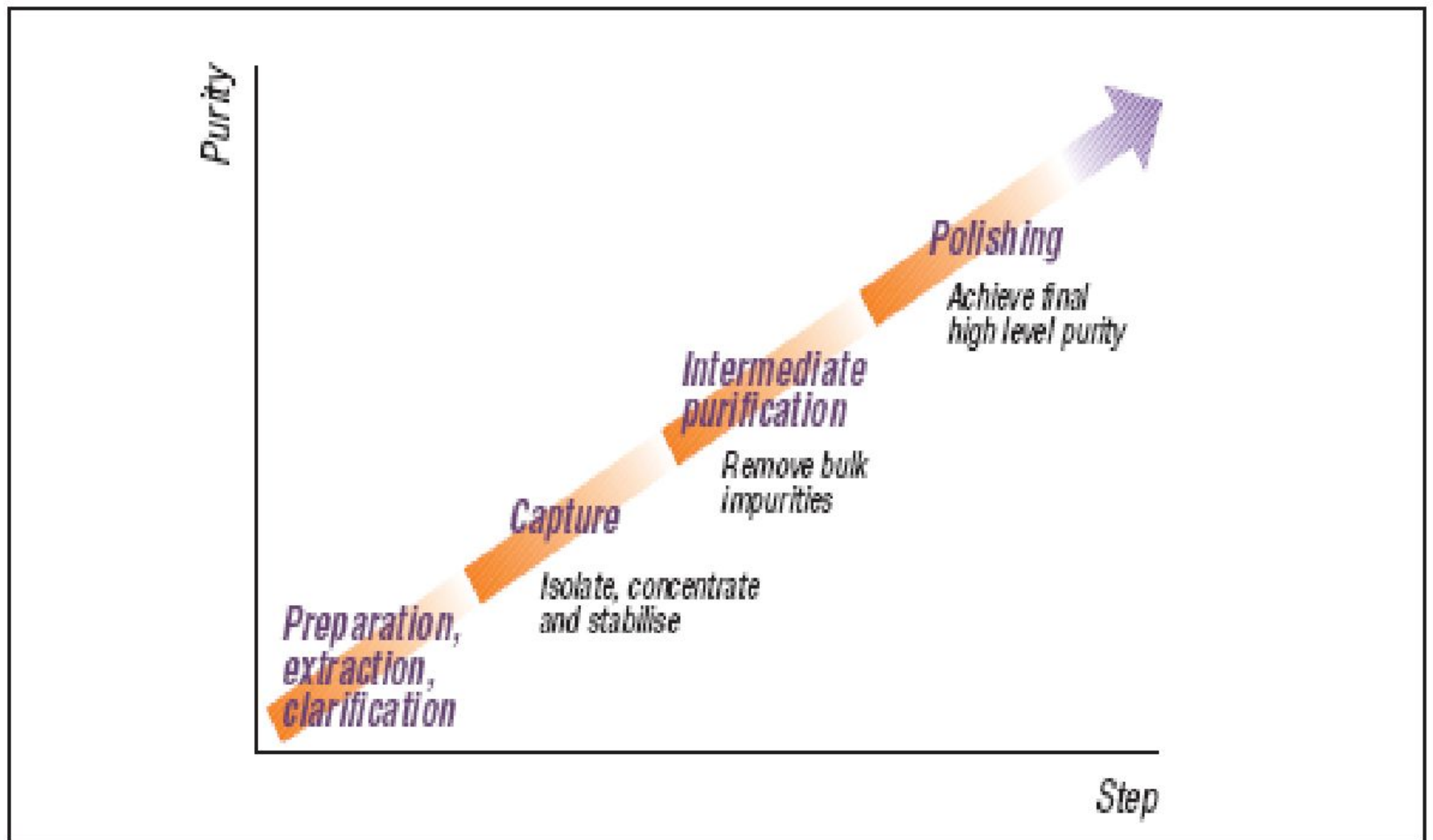


Fig. 3. Preparation and the Three Phase Purification Strategy.

Table 3. Protein properties used during purification.

Protein property	Technique
Charge	Ion exchange (IEX)
Size	Gel filtration (GF)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)
Charge, ligand specificity or hydrophobicity	Expanded bed adsorption (EBA) follows the principles of AC, IEX or HIC

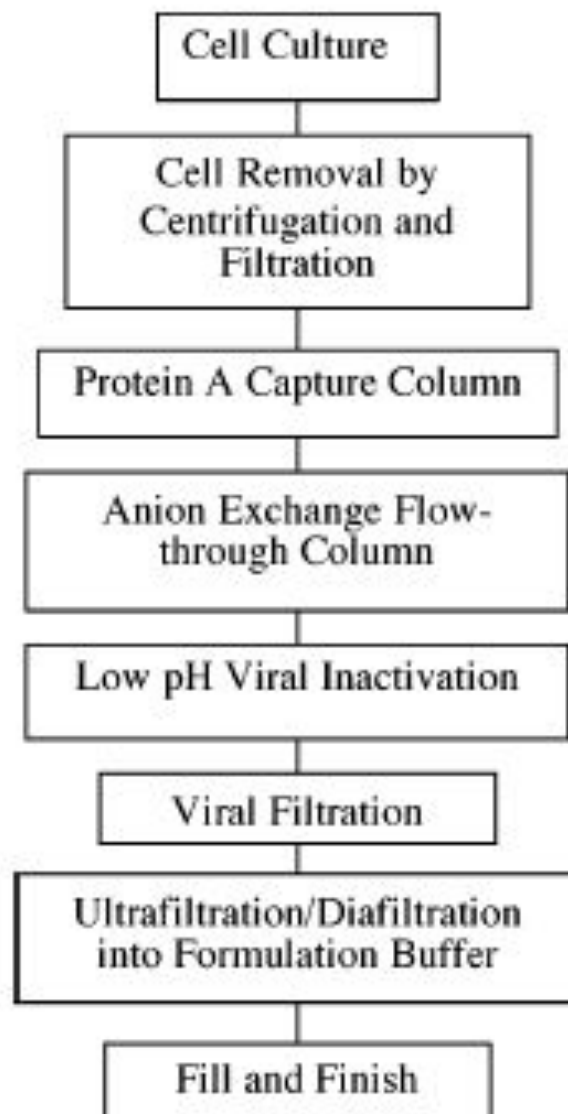


Figure 1. Minimal separation train for a mAb therapeutic.

Особенности экстракции - гомогенизации

Extraction process	Typical conditions	Protein source	Comment
<i>Gentle</i> Cell lysis (osmotic shock)	2 volumes water to 1 volume packed pre-washed cells	erythrocytes, <i>E.coli</i> periplasm: intracellular proteins	lower product yield but reduced protease release
Enzymatic digestion	lysozyme 0.2 mg/ml, 37 °C, 15 mins.	bacteria: intracellular proteins	lab scale only, often combined with mechanical disruption
Hand homogenisation	follow equipment instructions	liver tissue	
Mincing (grinding)	"	muscle	
<i>Moderate</i> Blade homogeniser	follow equipment instructions	muscle tissue, most animal tissues, plant tissues	
Grinding with abrasive e.g. sand	"	bacteria, plant tissues	
<i>Vigorous</i> Ultrasonication or bead milling	follow equipment instructions	cell suspensions: intracellular proteins in cytoplasm, periplasm, inclusion bodies	small scale, release of nucleic acids may cause viscosity problems inclusion bodies must be resolubilised
Manton-Gaulin homogeniser	follow equipment instructions	cell suspensions	large scale only
French press	follow equipment instructions	bacteria, plant cells	
Fractional precipitation	see section on fractional precipitation	extracellular: secreted recombinant proteins, monoclonal antibodies, cell lysates	precipitates must be resolubilised

Способы гомогенизации и типы гомогенизаторов

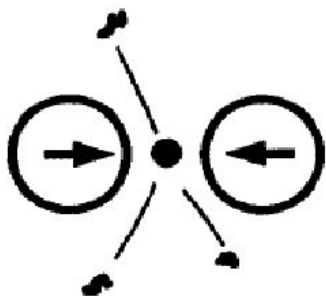


Figure 1 Schematic of bead mill action.



Figure 2 Schematic of rotor stator action.

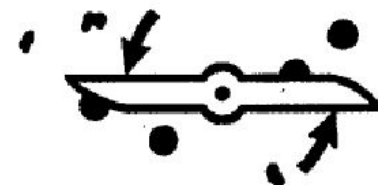


Figure 3 Schematic of blade blender action.

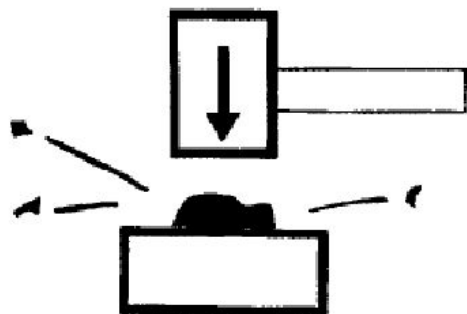


Figure 4 Schematic of freeze fracturing action.



Figure 5 Schematic of pestle and tube action.

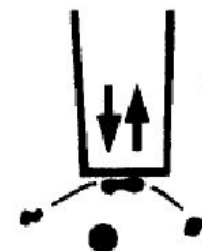


Figure 6 Schematic of sonication action.

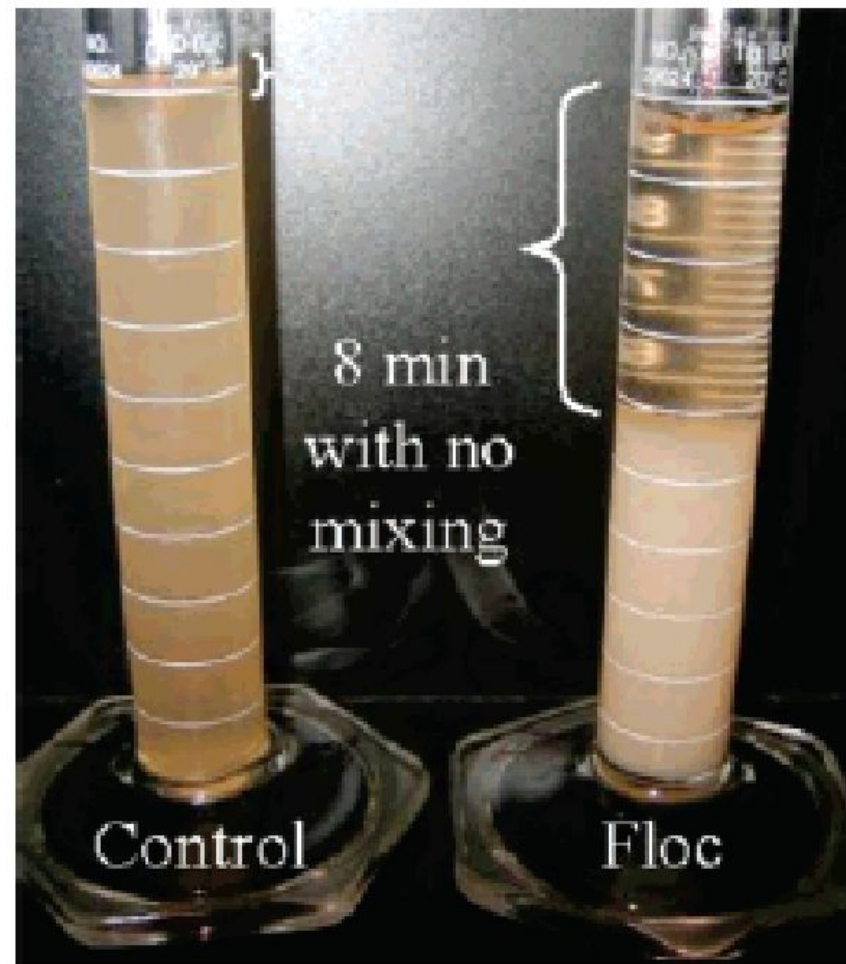


Figure 2. Flocculation of cellular debris by addition of nontoxic salts calcium chloride and potassium phosphate. (Copyright 2006 Wyeth Biopharma. Reproduced with permission.)

Plasma pre-treatment for ion exchange chromatography

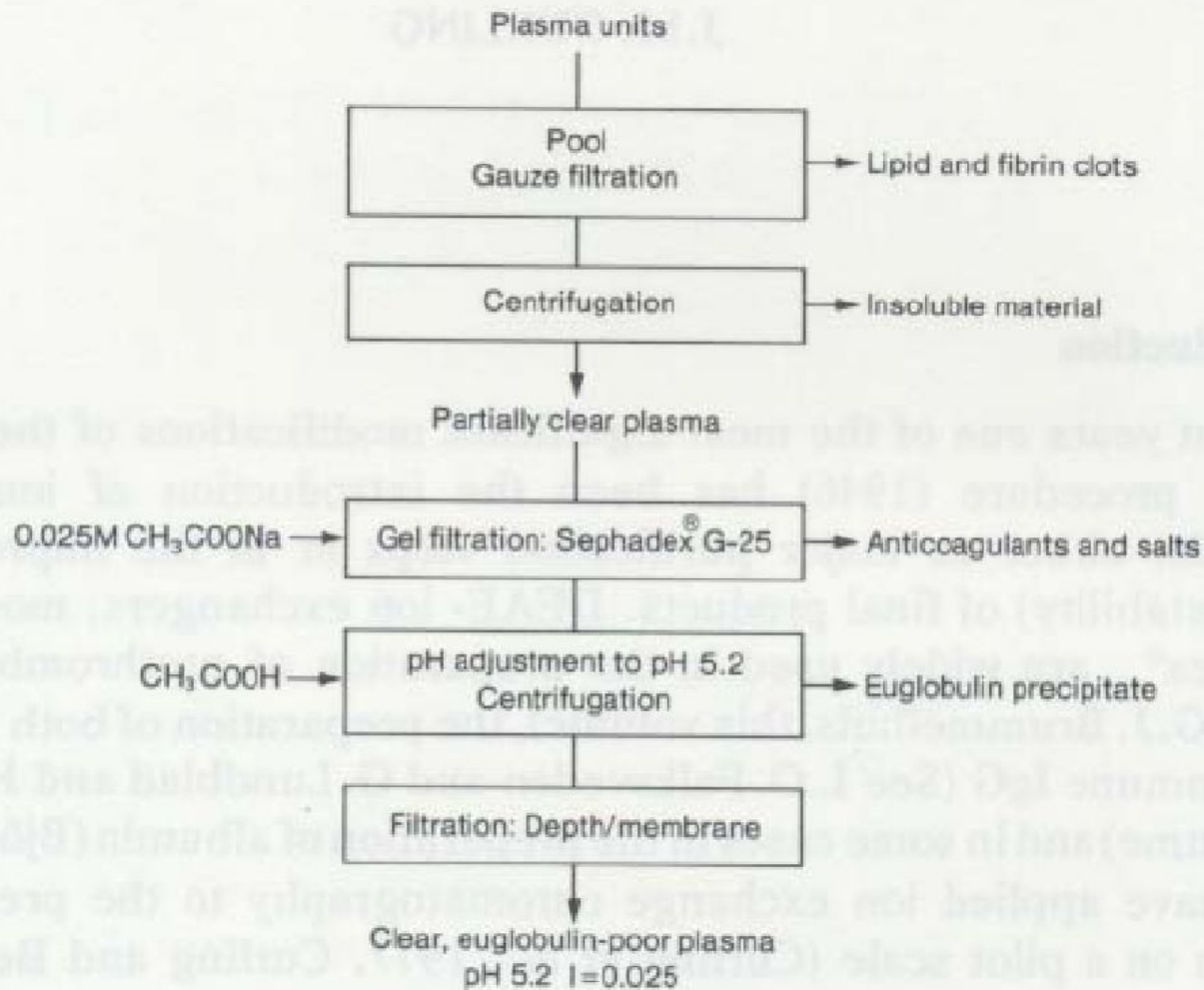


fig. 1. Plasma pre-treatment sequence for ion exchange chromatography.

	Typical conditions for use	Purpose
<i>Buffer components</i> Tris	20 mM, pH 7.4	maintain pH, minimise acidification caused by lysosomal disruption
NaCl	100 mM	maintain ionic strength of medium
EDTA	10 mM	reduce oxidation damage, chelate metal ions
Sucrose or glucose	25 mM	stabilise lysosomal membranes, reduce protease release
Detergents Ionic or non-ionic detergents	See Table 10	extraction and purification of integral membrane proteins solubilisation of poorly soluble proteins
DNase and RNase	1 µg/ml	degradation of nucleic acids, reduce viscosity of sample solution
<i>Protease inhibitors*</i>		<i>Inhibits</i>
PMSF	0.5 - 1 mM	serine proteases
APMSF	0.4 - 4 mM	serine proteases
Benzamidine-HCl	0.2 mM	serine proteases
Pepstatin	1 µM	aspartic proteases
Leupeptin	10 - 100 µM	cysteine and serine proteases
Chymostatin	10 - 100 µM	chymotrypsin, papain, cysteine proteases
Antipain-HCl	1 - 100 µM	papain, cysteine and serine proteases
EDTA	2 - 10 mM	metal dependent proteases, zinc and iron
EGTA	2 - 10 mM	metal dependent proteases e.g. calcium
<i>Reducing agents</i> 1,4 dithiothreitol, DTT	1 - 10 mM	keep cysteine residues reduced
1,4 dithioerythritol, DTE	1 - 10 mM	"
Mercaptoethanol	0.05%	"
<i>Others</i> Glycerol	5 - 10%	for stabilisation, up to 50% can be used if required

PMSF - Phenylmethylsulfonyl fluoride

APMSF - 4-Aminophenyl-methylsulfonyl fluoride

PMSF is a hazardous chemical. Half-life time in aqueous solution is 35 min. PMSF is usually stored as 10 mM or 100 mM stock solution (1.74 or 17.4 mg/ml in isopropanol) at - 20° C.

* Protease inhibitors are available in pre-made mixes from several suppliers.

Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, J-C. Janson and L. Rydén, 1998, 2nd ed. Wiley VCH and other sources.

Sodium dodecyl sulphate	0.1 - 0.5%	denatures proteins, used for SDS-PAGE use non-ionic detergents to avoid denaturation
Triton™ X-100	0.1 %	non-ionic detergent for membrane solubilisation. Note: <i>may absorb strongly at 280 nm!</i>
NP-40	0.05 - 2%	"
Dodecyl β D-maltoside	1%	"
Octyl β D-glucoside	1 - 1.5%	"

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulphate	as described	>1mg/ml proteins especially immunoglobulins	stabilizes proteins, no denaturation, supernatant can go directly to HIC
Dextran sulphate	as described	samples with high levels of lipoprotein, e.g ascites	precipitates lipoprotein
Polyvinylpyrrolidone	Add 3% (w/v), stir 4 hours, centrifuge, discard pellet	"	alternative to dextran sulphate
Polyethylene glycol (PEG, M.W. >4000)	up to 20% wt/vol	plasma proteins	no denaturation, supernatant goes direct to IEX or AC. Complete removal may be difficult
Acetone	up to 80% vol/vol at 0 °C	useful for peptide precipitation or concentration of sample for electrophoresis	may denature protein irreversibly
Polyethyleneimine	0.1% w/v		precipitates aggregated nucleoproteins
Protamine sulphate	1%		"
Streptomycin sulphate	1%		precipitation of nucleic acids

Details taken from Protein Purification, Principles and Practice, R.K. Scopes. 1994, Springer., Protein Purification, Principles, High Resolution Methods and Applications, J-C. Janson and L. Rydén, 1998, 2nd ed. Wiley VCH and other sources

Most Lyotropic ("Salting Out")

PO_4^{3-}	NH_4^+
SO_4^{2-}	Rb^+
CH_3COO^-	K^+
Cl^-	Na^+
Br^-	Cs^+
NO_3^-	Li^+
ClO_4^-	Mg^{2+}
I^-	Ca^{2+}
SCN^-	Ba^{2+}

Most Chaotropic

Values calculated according to Protein Purification, R. K. Scopes (Springer-Verlag, New York), Third Edition, p. 346, 1993.

Starting percent saturation	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Amount of ammonium sulphate to add (grams) per liter of solution at 20 °C																
0	113	144	178	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	148	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	20	50	80	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	20	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	258	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

For further details showing variation of % saturation versus temperature and a review of precipitation techniques see Guide to Protein Purification, Methods in Enzymology, Vol. 182, p. 291 Academic Press 1990.

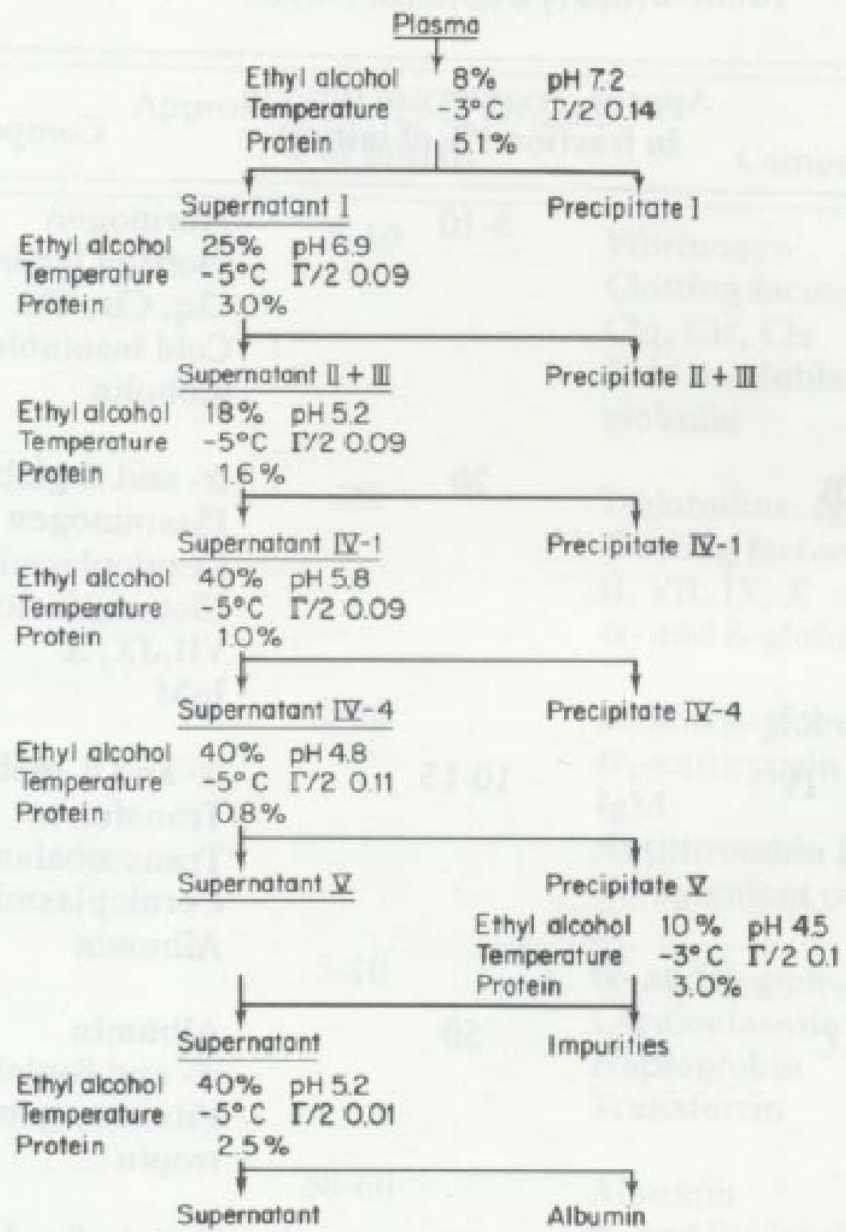


Fig. 6. Method 6 (Cohn *et al.*, 1946).

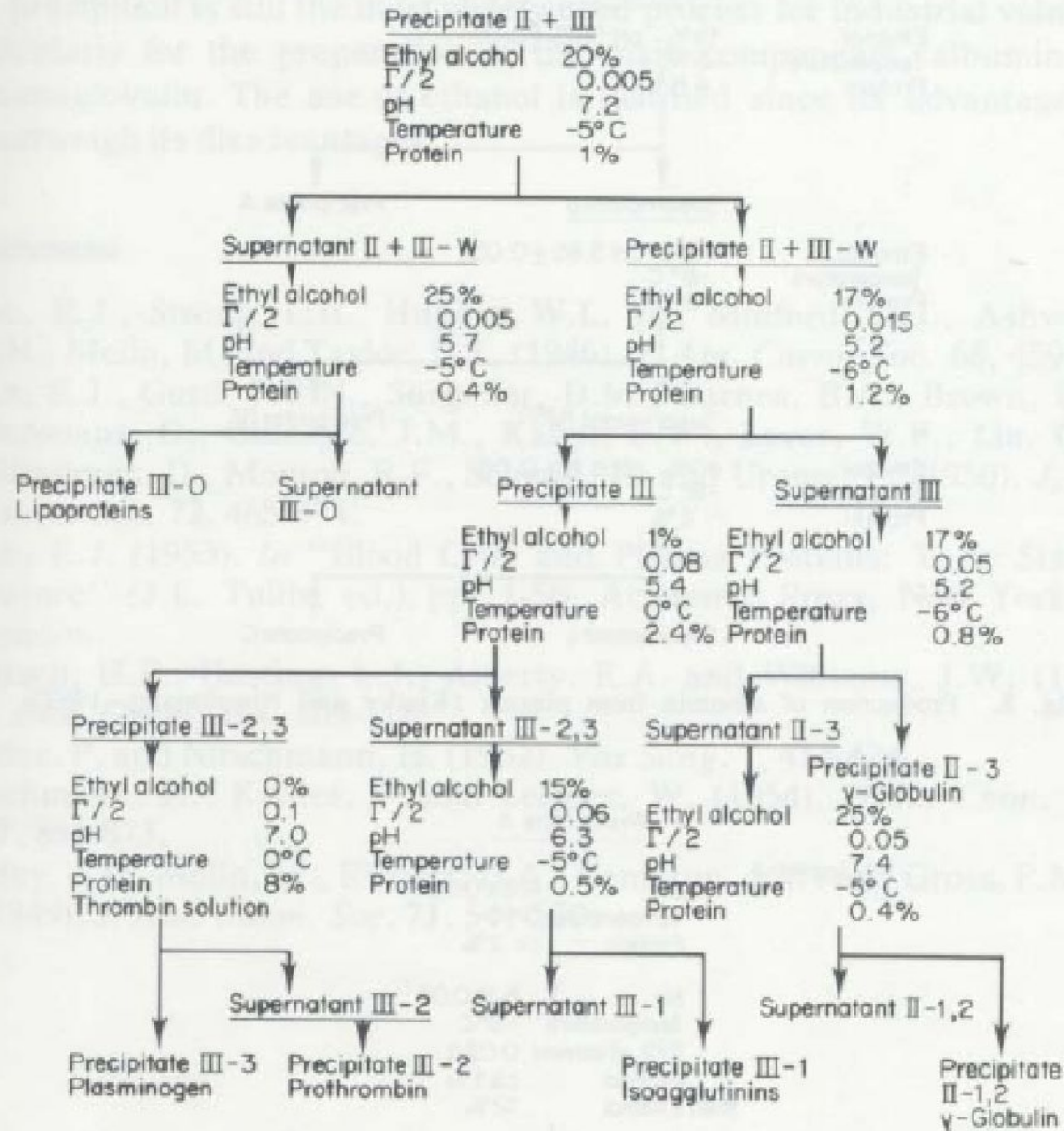


Fig. 7. Method 9 (Oncley *et al.*, 1949).

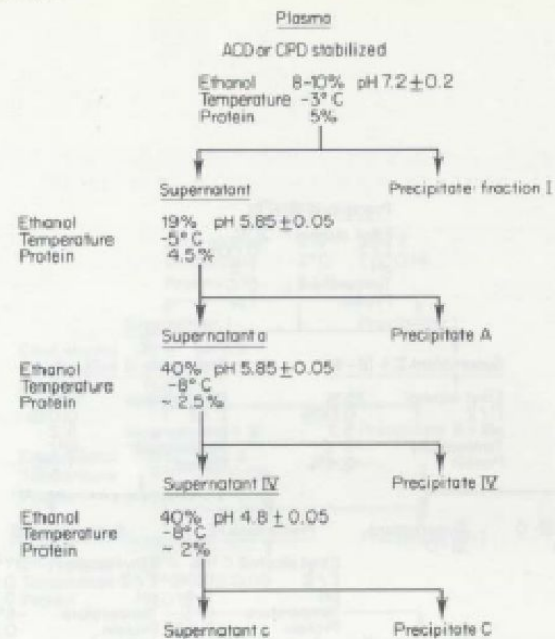


Fig. 8. Production of albumin from plasma (Kistler and Nitschmann, 1962).

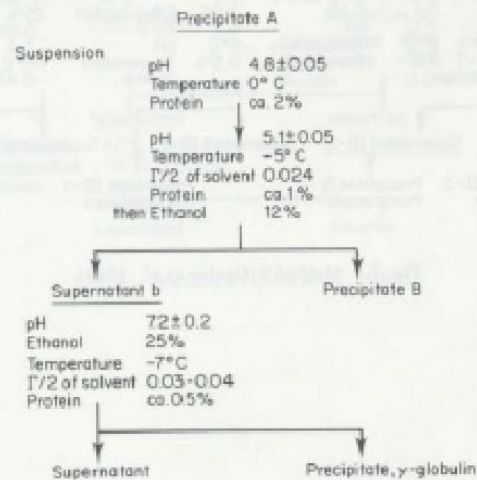


Fig. 9. Processing of precipitate A to γ -globulin (Kistler and Nitschmann, 1962).

TABLE 1

Distribution of Plasma Proteins in PEG Fractions

Plasma Proteins ^a	Starting Cryosup. mg/100ml	% Distribution			
		A(0-4%)	B(4-10%)	C(10-20%)	D(20% sup.)
Total Protein	6100	4	21	12	62
Fibrinogen	179	68	20	—	—
Plasminogen	16	25	44	19	—
α_1 -Antitrypsin	166	1	3	5	88
Antithrombin III	30	—	—	23	77
α_2 -Macroglobulin	189	—	35	65	—
C3 Component	56	7	86	7	—
Ceruloplasmin	22	—	14	23	73
Haptoglobin	108	1	1	40	56
Albumin	3440	3	3	4	86
α_1 -Acid Glycoprotein	54	—	—	—	100
Transferrin	223	1	5	22	58
IgA	157	2	32	58	20
IgG	740	3	85	15	1
IgM	114	7	31	—	—
	Reciprocal Titres ^b				
Prothrombin	8	—	25	50	25
β -Lipoprotein	16	—	100	—	—
α -Lipoprotein	64	2	13	25	50
C1 Inactivator	4	—	—	—	100
HB _s Ag ^c	1024	3	13	100	—

^aQuantitative determination by Mancini radial immunodiffusion technique

^bSemiquantitative estimate by Ouchterlony immunoprecipitation technique

^cRadioimmunoassay

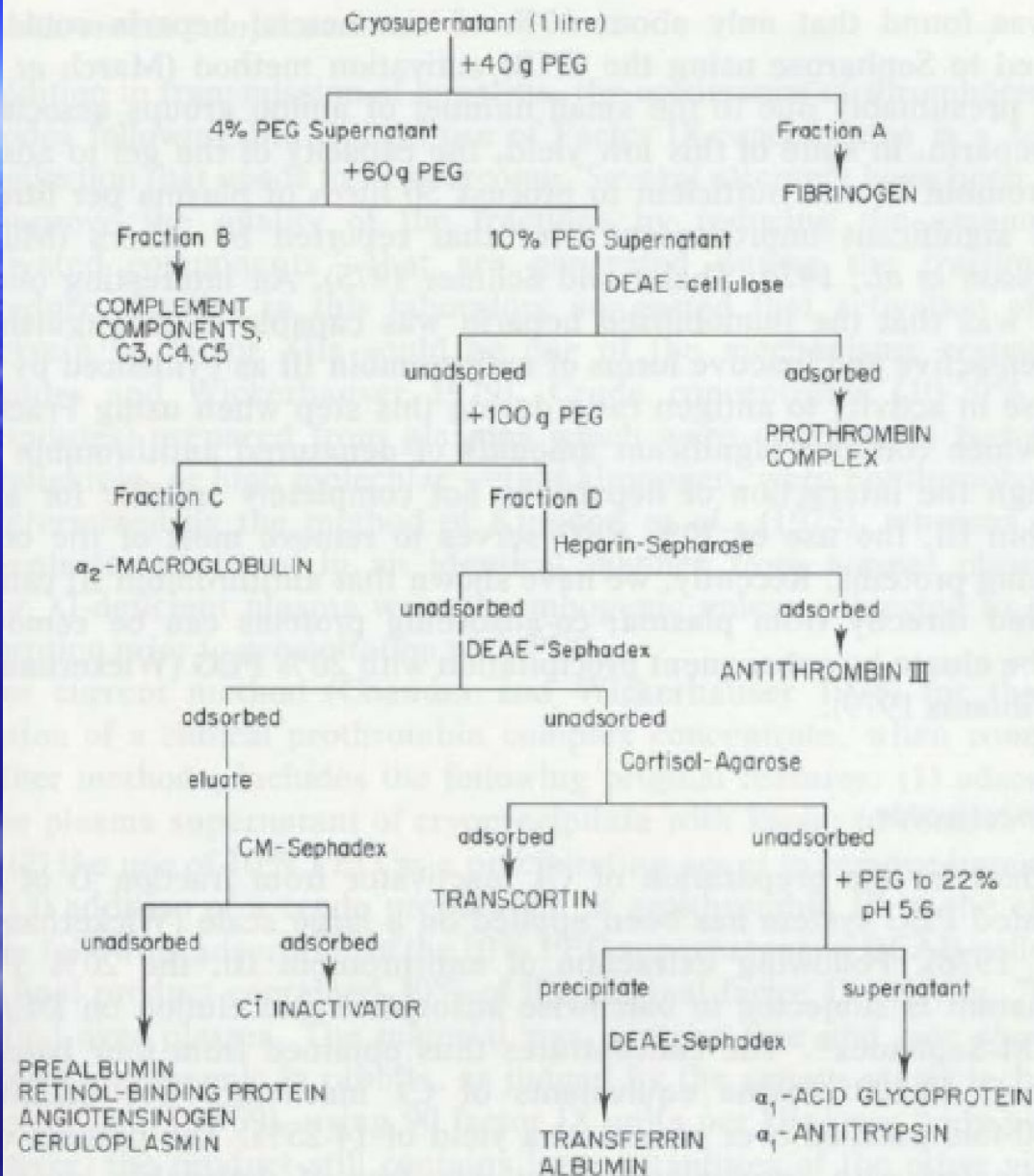


Fig. 1. Isolation of various proteins from PEG fractions of human plasma.

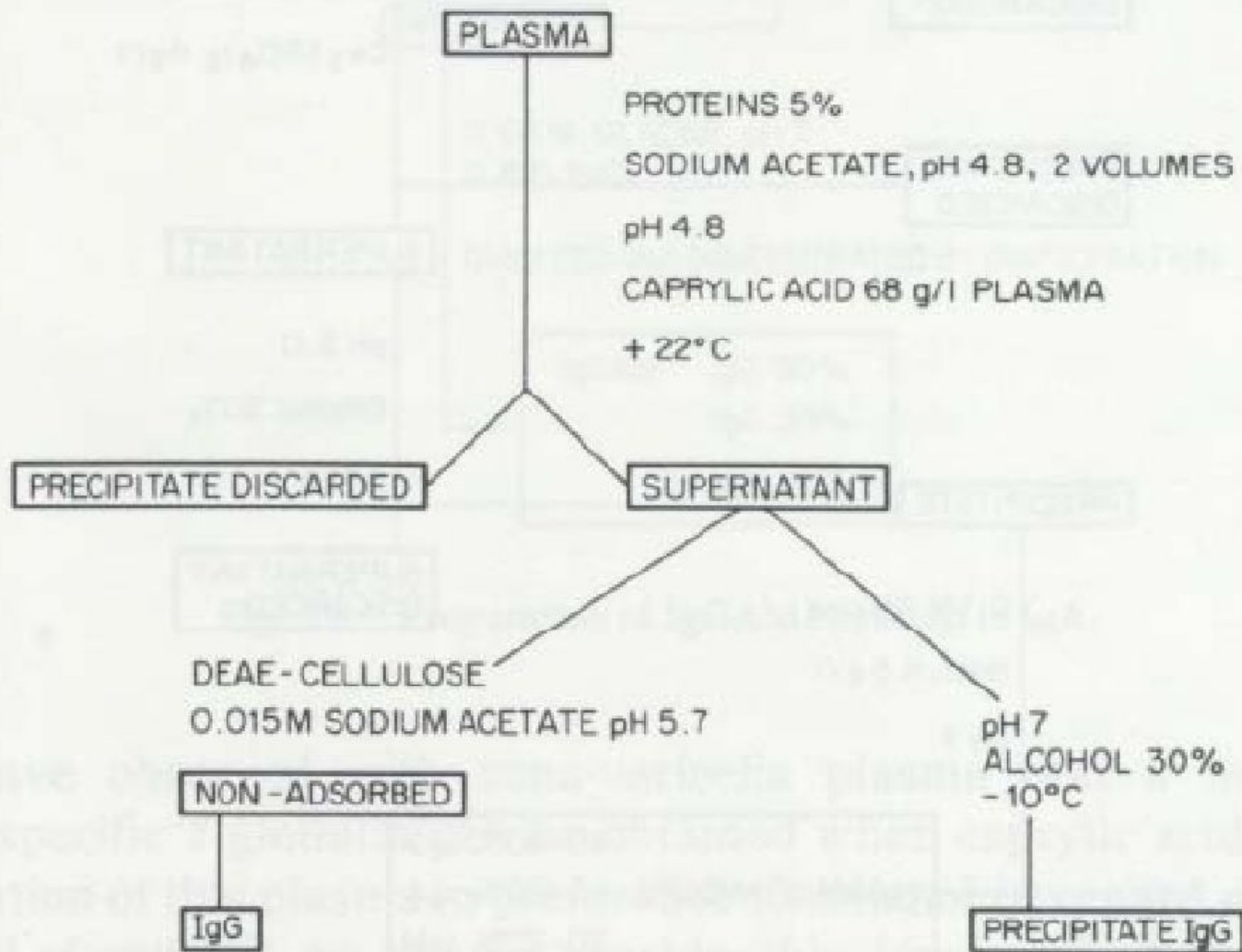
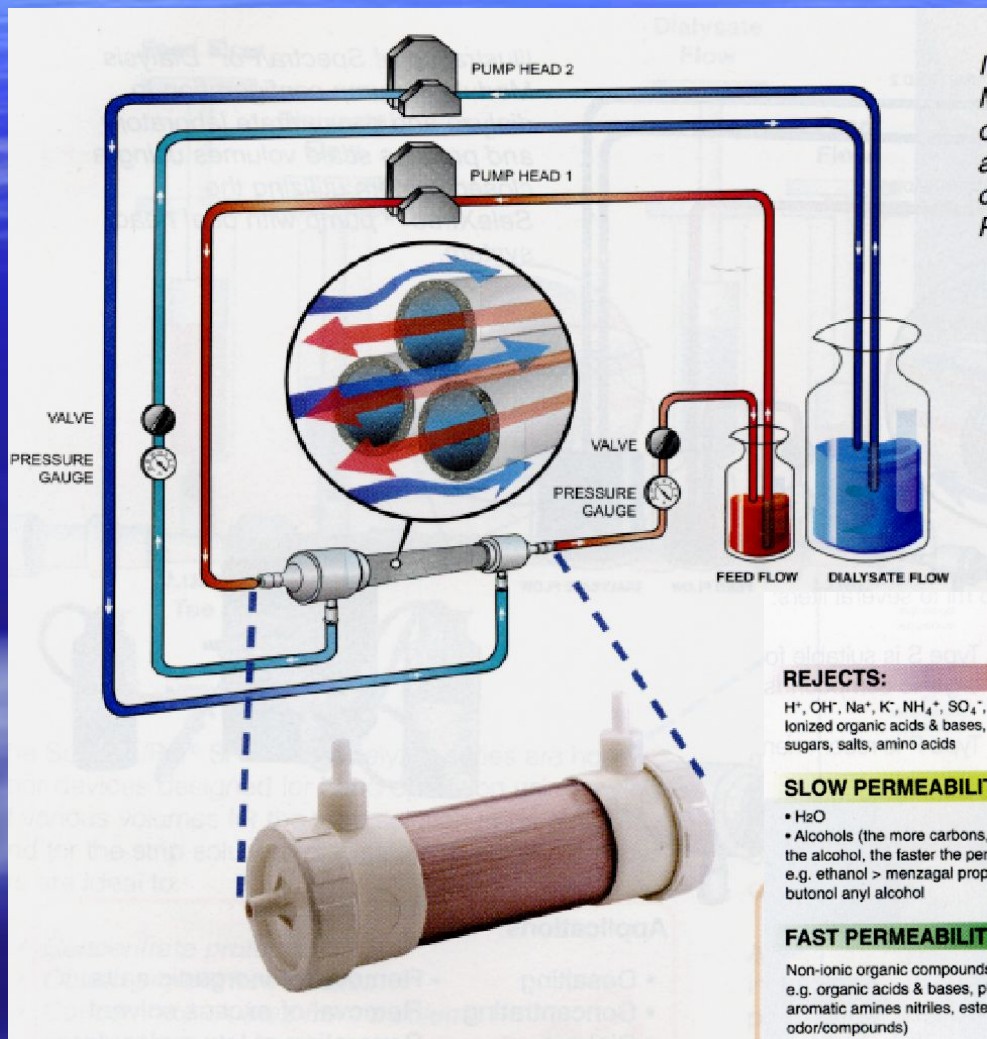


Fig. 16. Purification of IgG by caprylic acid precipitation.

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 - 8 M	remove using Sephadex G-25
Guanidine hydrochloride	3 - 8 M	remove using Sephadex G-25 or during IEX
Triton X-100	2%	"
Sarcosyl	1.5%	"
N-octyl glucoside	2%	"
Sodium dodecyl sulphate	0.1 - 0.5%	exchange for non-ionic detergent during first chromatographic step, avoid anion exchange chromatography
alkaline pH	> pH 9, NaOH	may need to adjust pH during chromatography to maintain solubility

Концентрирование, диализ и смена буфера в установке на полых волокнах



REJECTS:

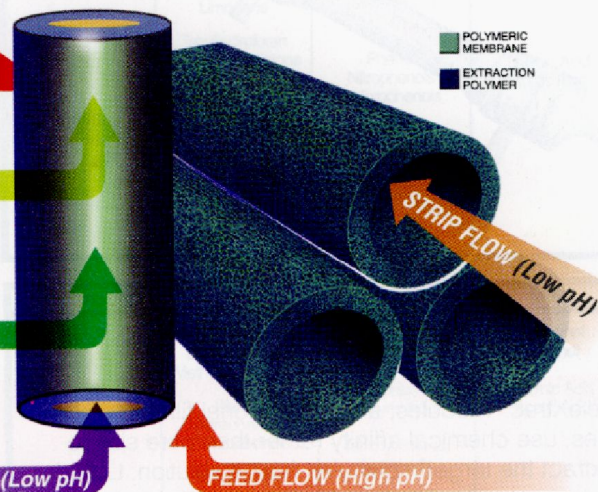
H^+ , OH^- , Na^+ , K^+ , NH_4^+ , SO_4^- , NO_3^- ,
ionized organic acids & bases, Zwitterions,
sugars, salts, amino acids

SLOW PERMEABILITY

- H_2O
- Alcohols (the more carbons, i.e. larger the alcohol, the faster the permeability)
e.g. ethanol > menzagal propanol > butonol anyl alcohol

FAST PERMEABILITY

Non-ionic organic compounds,
e.g. organic acids & bases, phenolics,
aromatic amines nitriles, esters (flavor/
odor/compounds)



Концентрирование в установке на полых волокнах

MiniKros® Sampler System Schematic of Sampler System Ordering Information

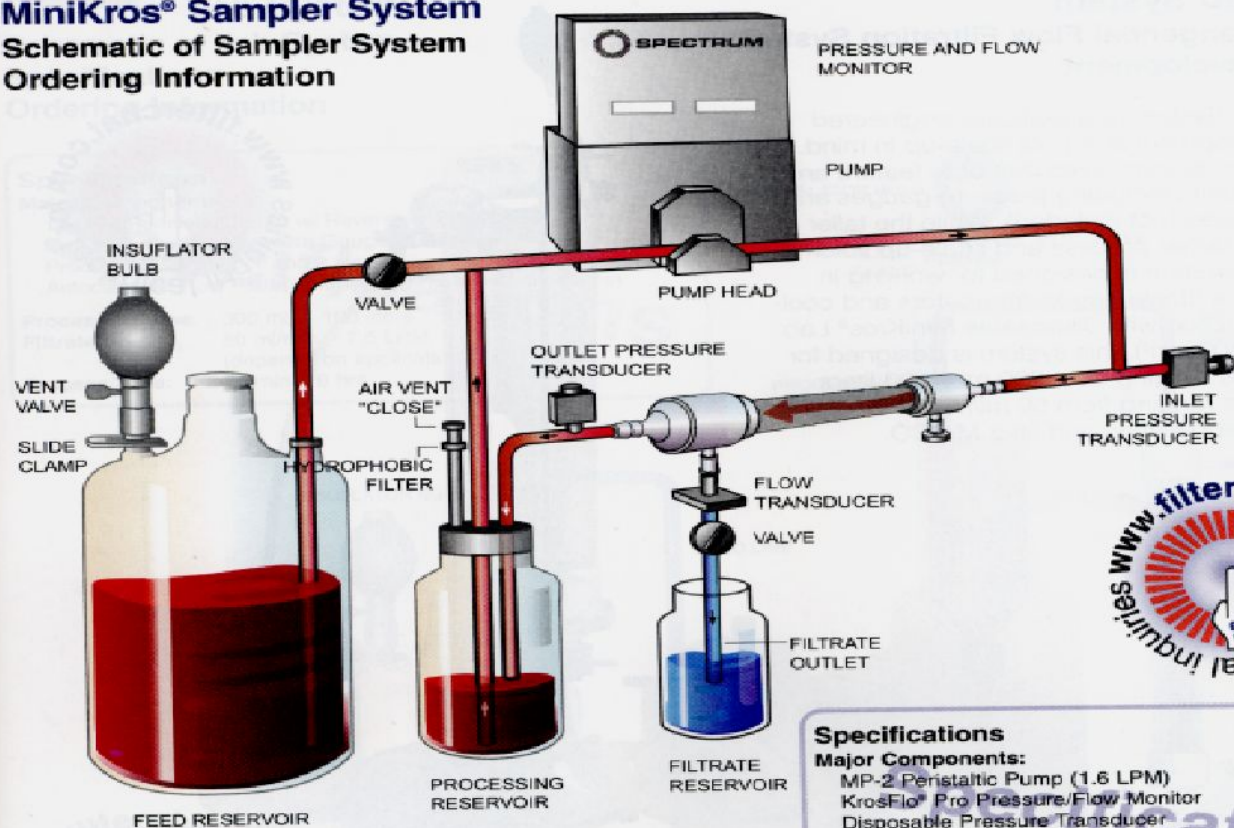


Diagram of MiniKros® Sampler System

Specifications

Major Components:

- MP-2 Peristaltic Pump (1.6 LPM)
- KrosFlo® Pro Pressure/Flow Monitor
- Disposable Pressure Transducer
- 60 ml Process Reservoir
- 2 liter Feed Reservoir
- Disposable Flow Path (tubing and connections)

Process Volume:	50 ml – 3 liters
Filtrate Rate:	10 – 1,000 ml/min
Process Time:	30 min to 6 hrs

