HPLC '90

Poster Presentation

A new systematic approach for the development of protein separations

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Abstract

Advances in the development of high resolution chromatographic columns have greatly improved the quality of protein separations from complex biological matrices. Historically, these columns have been used later in the purification process, typically as a polishing step. However, the early steps, generally gross precipitation followed by open column purification, are time-consuming and often give poor recovery with fairly small improvements in homogeneity. The use of high resolution columns early in the purification process can give better yield with higher specific activity. We have developed a systematic approach for screening columns and buffer combinations for use with crude samples. This screening technique will be demonstrated for lactate dehydrogenase isolated from crude extracts of mouse liver.

INTRODUCTION

Protein purification is a multi-step process exploiting a range of physical and chemical separation technologies. Most commonly, the isolation begins with gross precipitation techniques to achieve some mass and volume reduction, followed by one or more relatively low resolution open column separations. Typically, modern, high resolution columns are only used at the final step. Both recovery and the degree of purification should be better if such columns could be used earlier in the procedure, particularly if the separation conditions were optimized for the paricular protein and biological matrix.

Ion-exchange chromatography is recognized as a generally powerful tool for protein isolation. The separations are usually effected with an ionic strength gradient. The pH is selected to be near the isoelectric point, if known, or to give relatively strong retention based on experience. The only optimization commonly exploited is adjustment of the gradient slope. It is recognized that careful adjustment of pH is a powerful tool for adjusting the selectivity of a protein separation, but it is time-consuming and difficult to systematically optimize this parameter.

The use of the Waters 650 Advanced Protein Purification System™ facilitates this process because it can blend buffers from four separate reservoirs. In the Auto-Blend™ Method, two reservoirs are used for concentrated stock buffers for adjustment of pH, and the remaining two reservoirs are used for 1M sodium chloride and water to generate ionic strength gradients independent of pH. By selecting different proportions of the buffer stocks, it is possible to generate any desired pH. When this system is coupled to a refrigerated autosampler, the separation of a given sample may be studied at a series of pH values. Such a study gives the optimimal conditions for a given sample and column and facilitates the comparison of different packing materials.

MATERIALS AND METHODS

Instrument:
- Waters 650 Advanced Protein Purification System
- Waters 490 Multi-Wavelength Detector or Waters 484 Tunable UV-VIS Dectector
- Waters 712R Refrigerated Autosampler
- Waters 860 Chromatography Control and Data Station

Chromatographic Materials:
- Protein-Pak™ DEAE 8HR, 10 X 100mm
- Protein-Pak™ DEAE 40HR, 10 X 100mm
- Accell™ QMA, 10 X 100mm

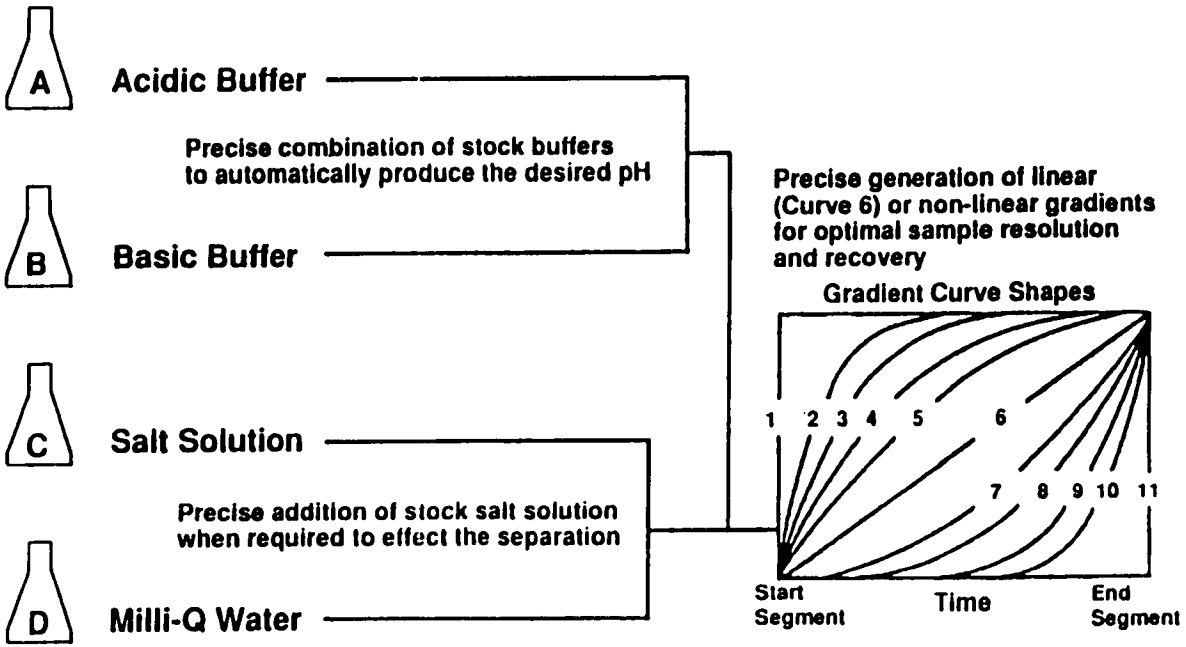
Buffers:
- A. 0.1M Tris-HCl
- B. 0.1M Tris Base
- C. 1.0M NaCl
- D. MilliQ™ Water

Operating Conditions:
- All separations at room temperature.
- All 10 X 100mm Columns at 70min cycle time
- Gradient conditions shown on chromatogram.

Sample Preparation:
- Livers harvested from freshly sacrificed mice.
- Homogenized and centrifuged at 48000g.
- 200µl injected on 10 X 100mm columns

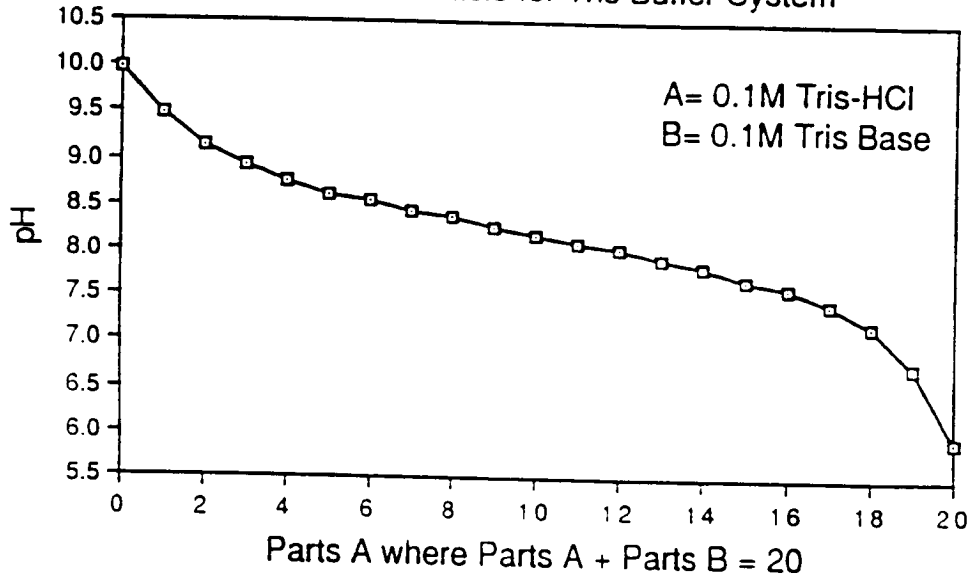
Assay Protocols:
- Lactate Dehydrogenase activity determined in 1mM pyruvate, 0.15mM NADH, 0.05M Sodium Phosphate, pH 7.0, with a sufficient aliquot of sample to give a ΔO.D.340 of 0.03 to 0.08/min. Results expressed as I.U., µmoles of NADH/min/ml of enzyme solution.
- Protein determined with Bio-Rad Protein Assay Kit, calibrated with ovalbumin.

WATERS AUTO-BLEND™ METHOD FOR OPTIMIZING PROTEIN SEPARATIONS

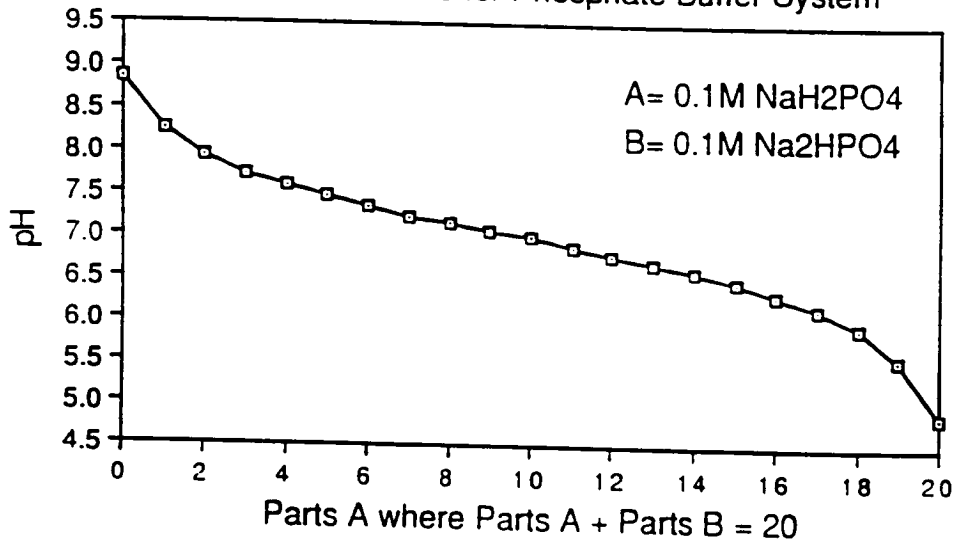


Waters Auto-Blend™ Tables For Tris and Phosphate

Auto-Blend™ Table for Tris Buffer System



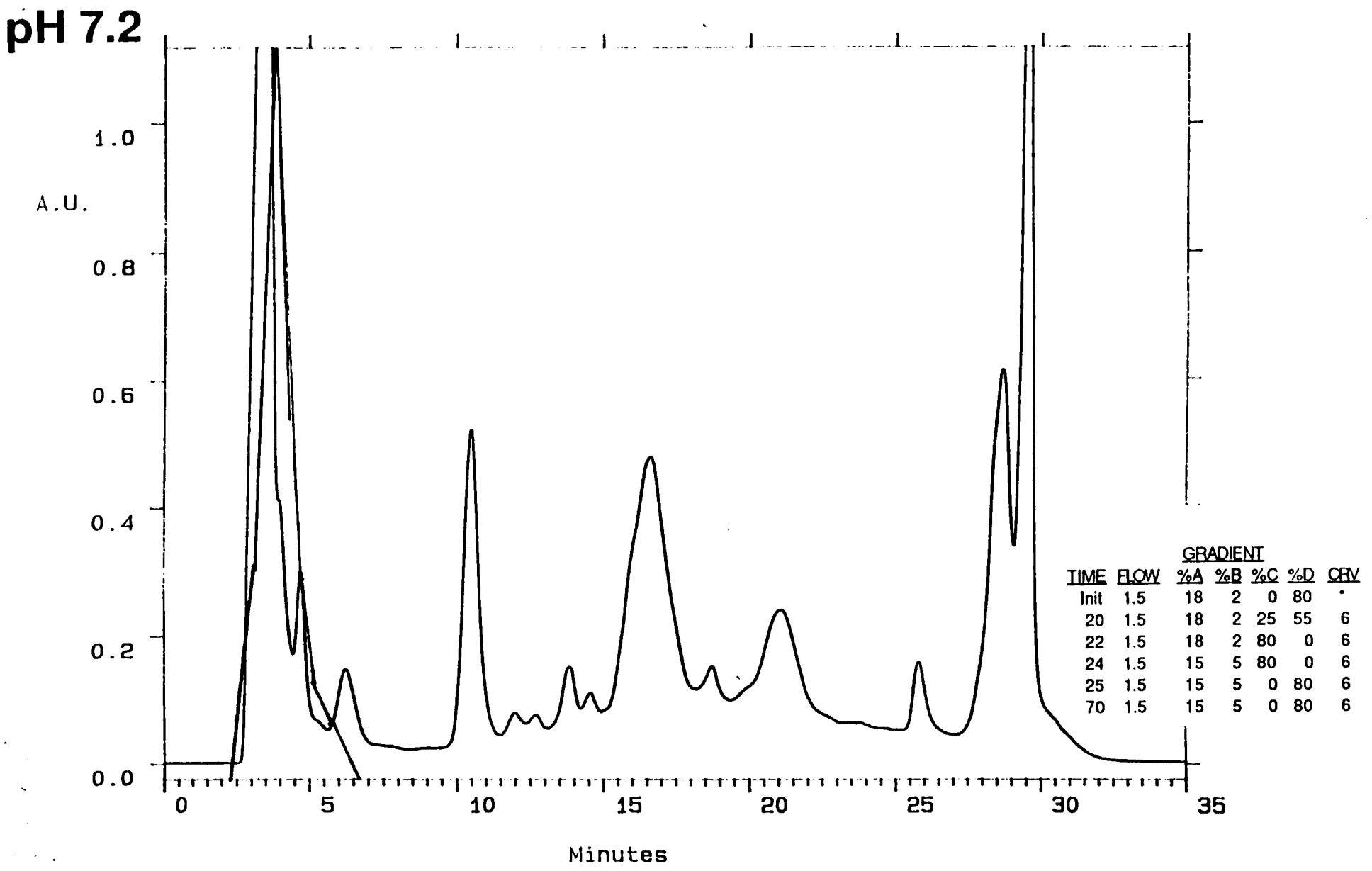
Auto-Blend™ Table for Phosphate Buffer System



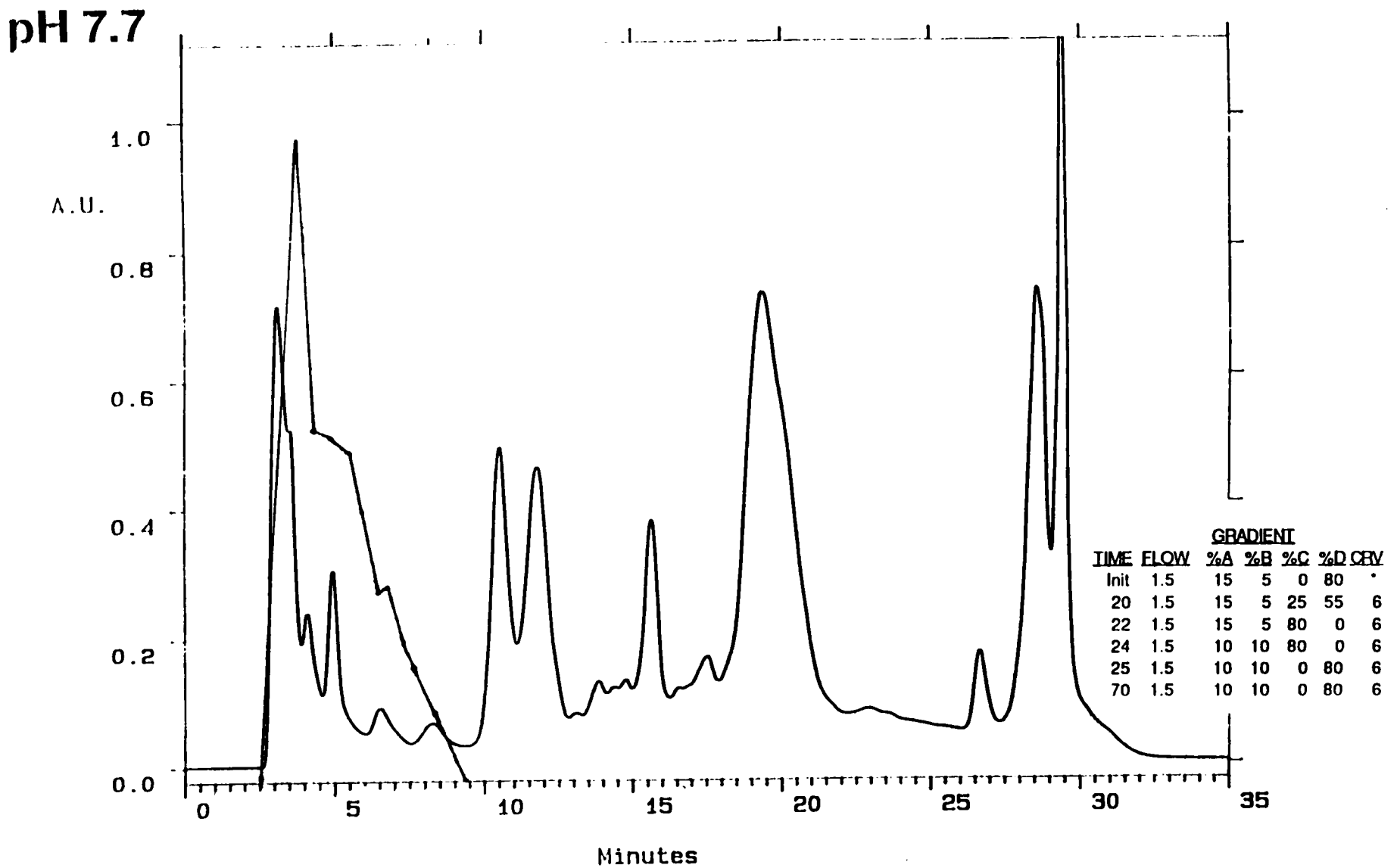
SPECIFIC ACTIVITY

	pH				
COLUMN	7.2	7.7	8.2	8.4	8.6
DEAE 8HR	9.69	9.94	19.33	23.14	9.28
DEAE 40HR				14.73	
ACCELL QMA	2.25	6.33	6.52	4.17	3.72

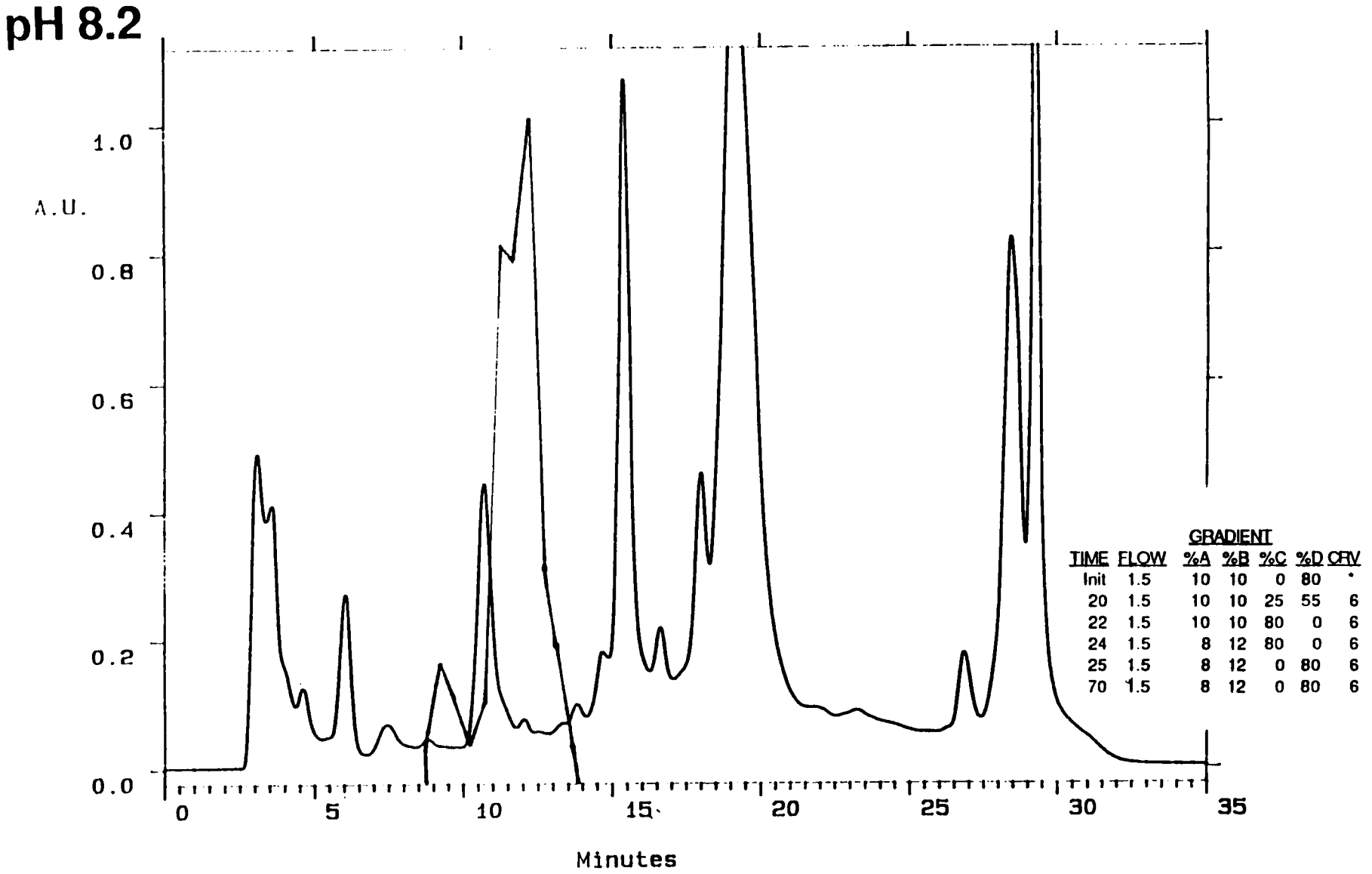
PROTEIN-PAK™ DEAE 8HR



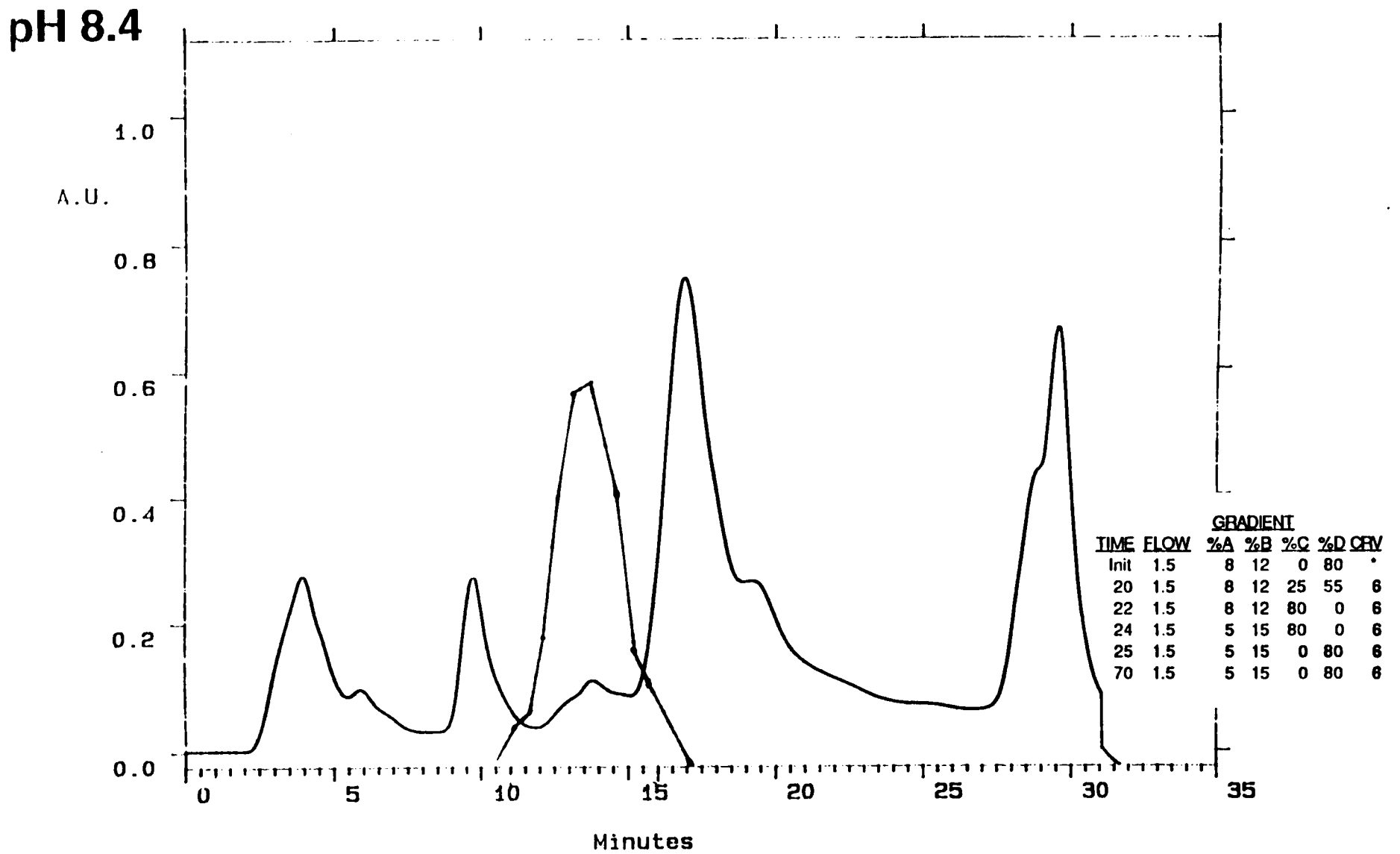
PROTEIN-PAK™ DEAE 8HR



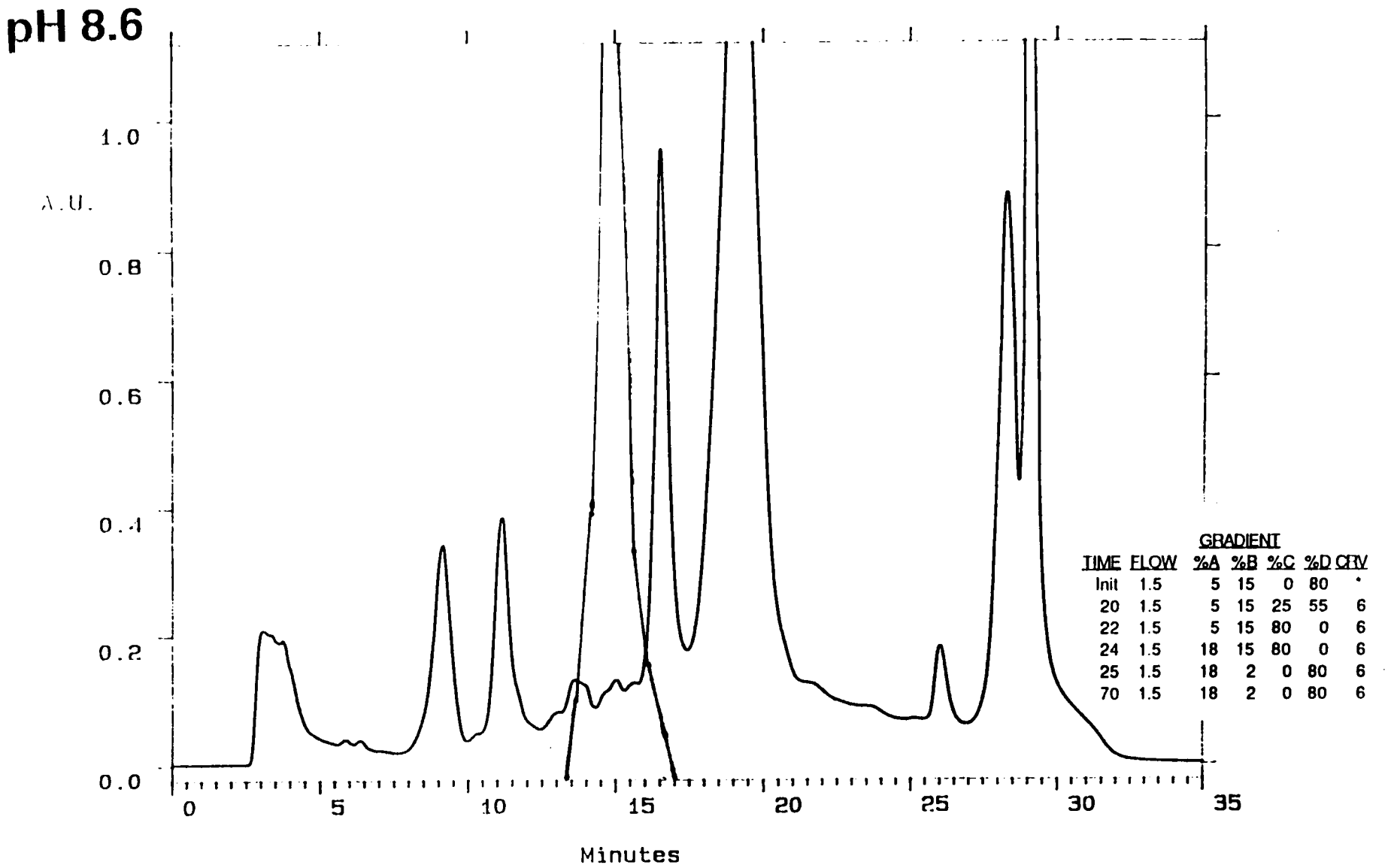
PROTEIN-PAK™ DEAE 8HR



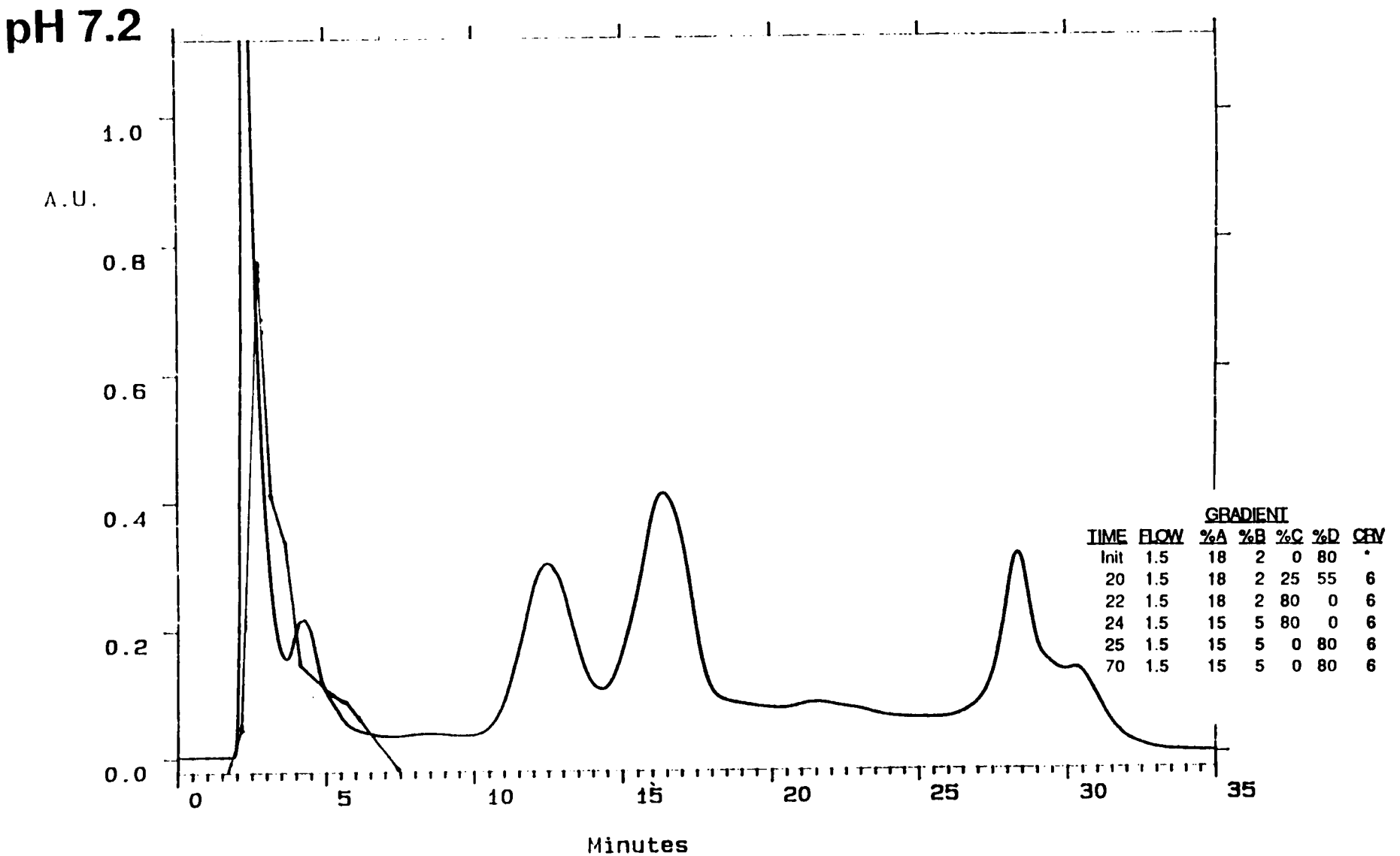
PROTEIN-PAK™ DEAE 40HR



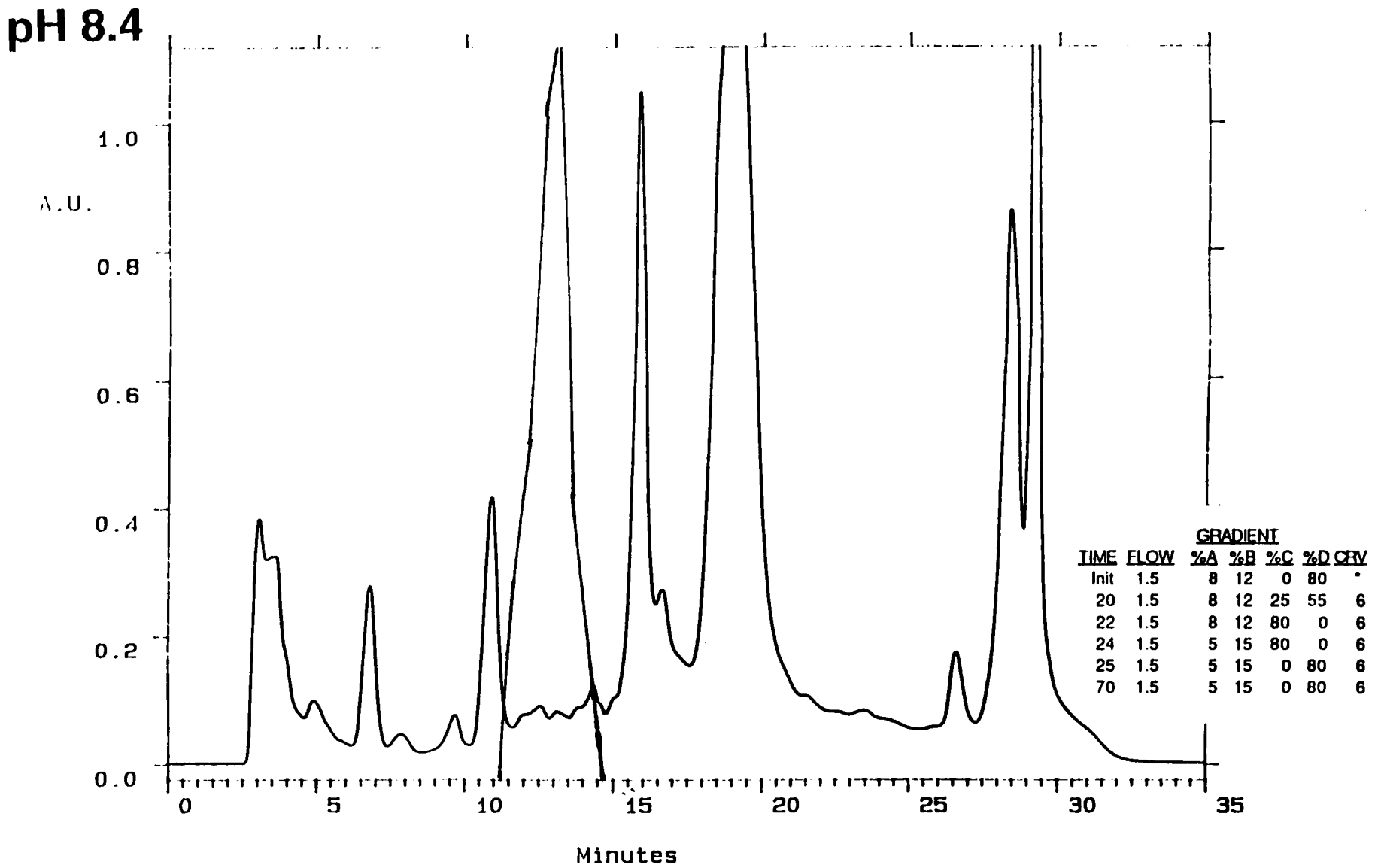
PROTEIN-PAK™ DEAE 8HR



ACCELL™ QMA

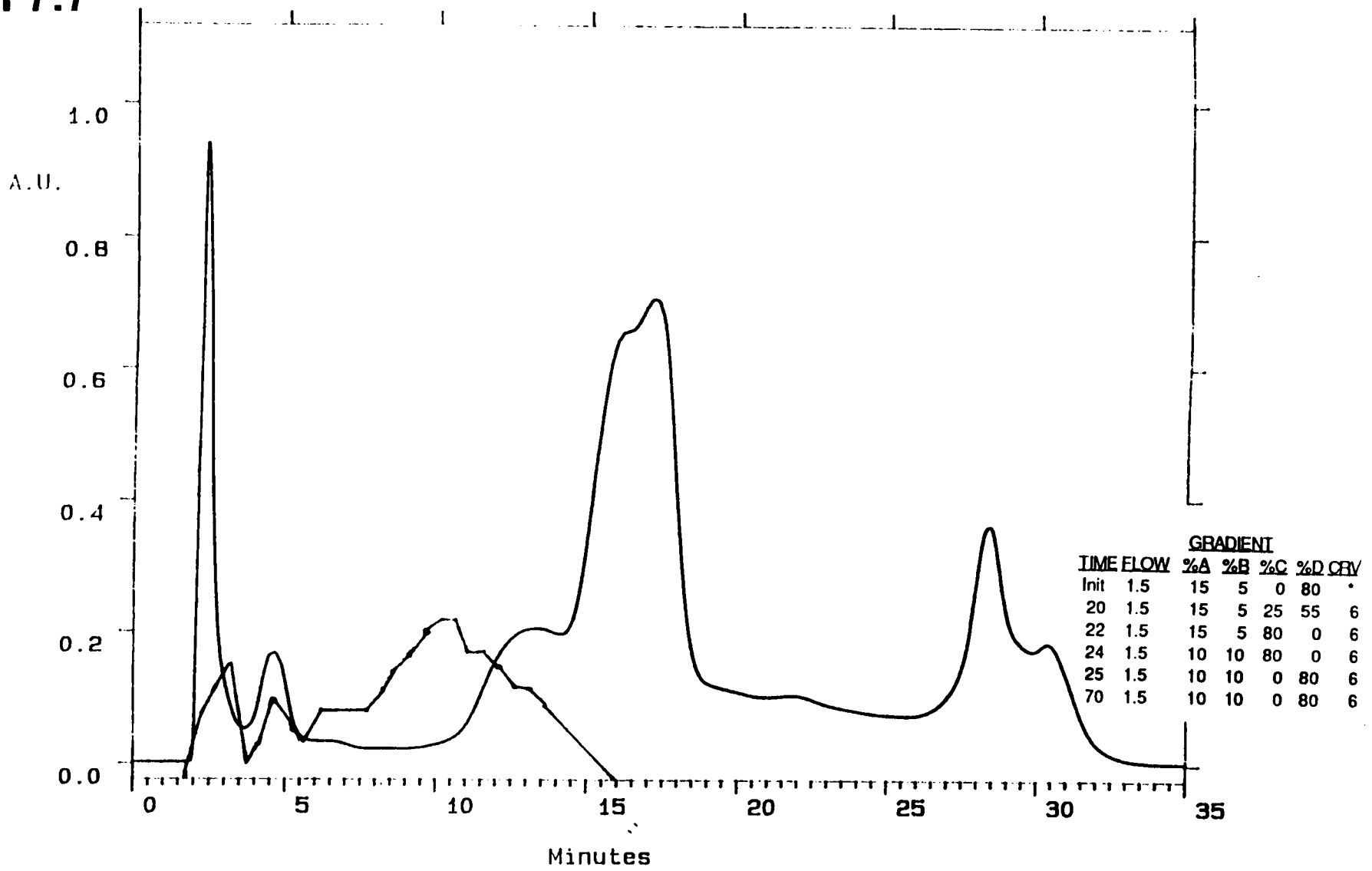

pH 7.2
A.U.
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Minutes
GRADIENT
TIME FLOW %A %B %C %D CRV
Init 1.5 18 2 0 80 *
20 1.5 18 2 25 55 6
22 1.5 18 2 80 0 6
24 1.5 15 5 80 0 6
25 1.5 15 5 0 80 6
70 1.5 15 5 0 80 6

PROTEIN-PAK™ DEAE 8HR



ACCELL™ QMA

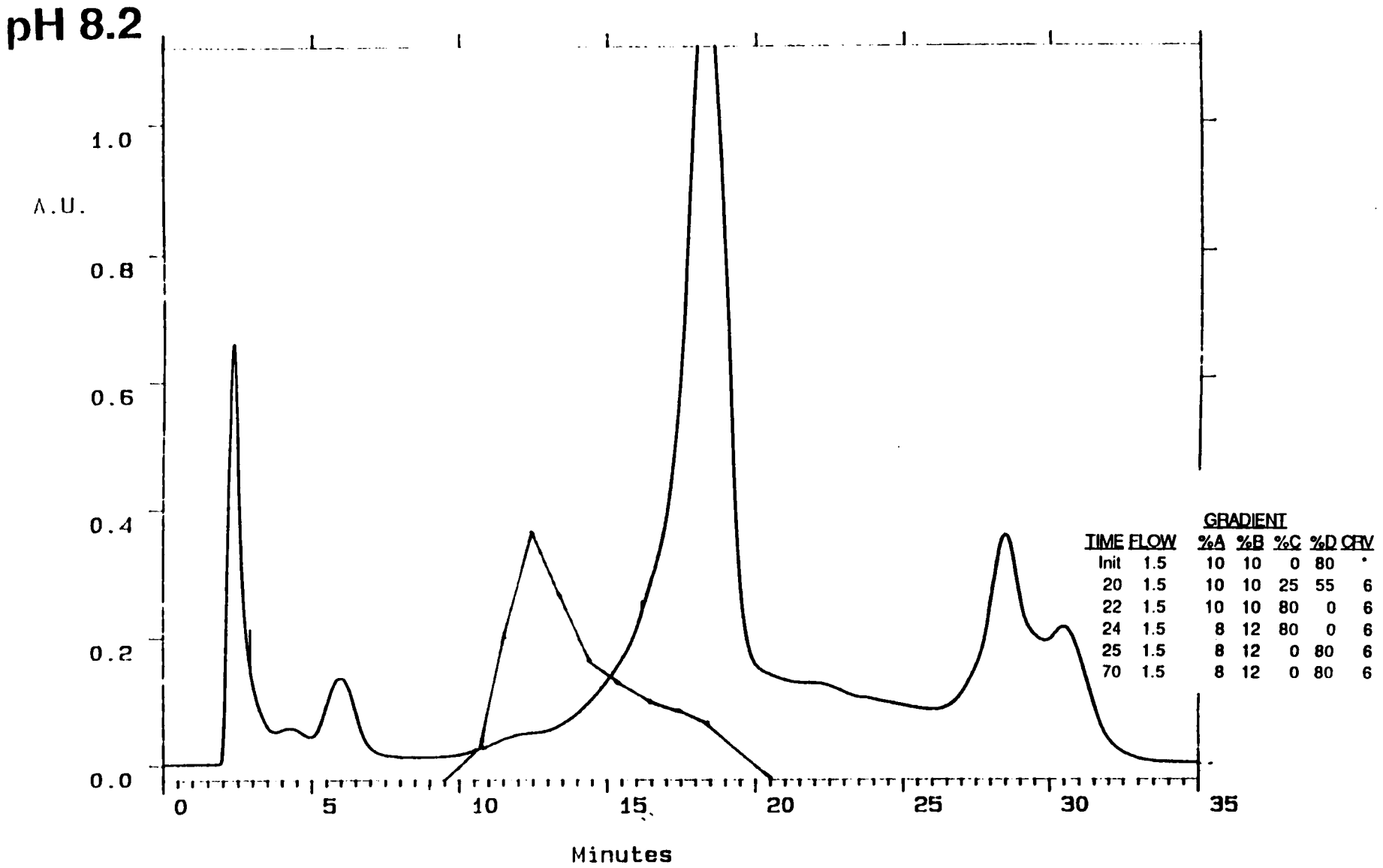
pH 7.7



GRADIENT

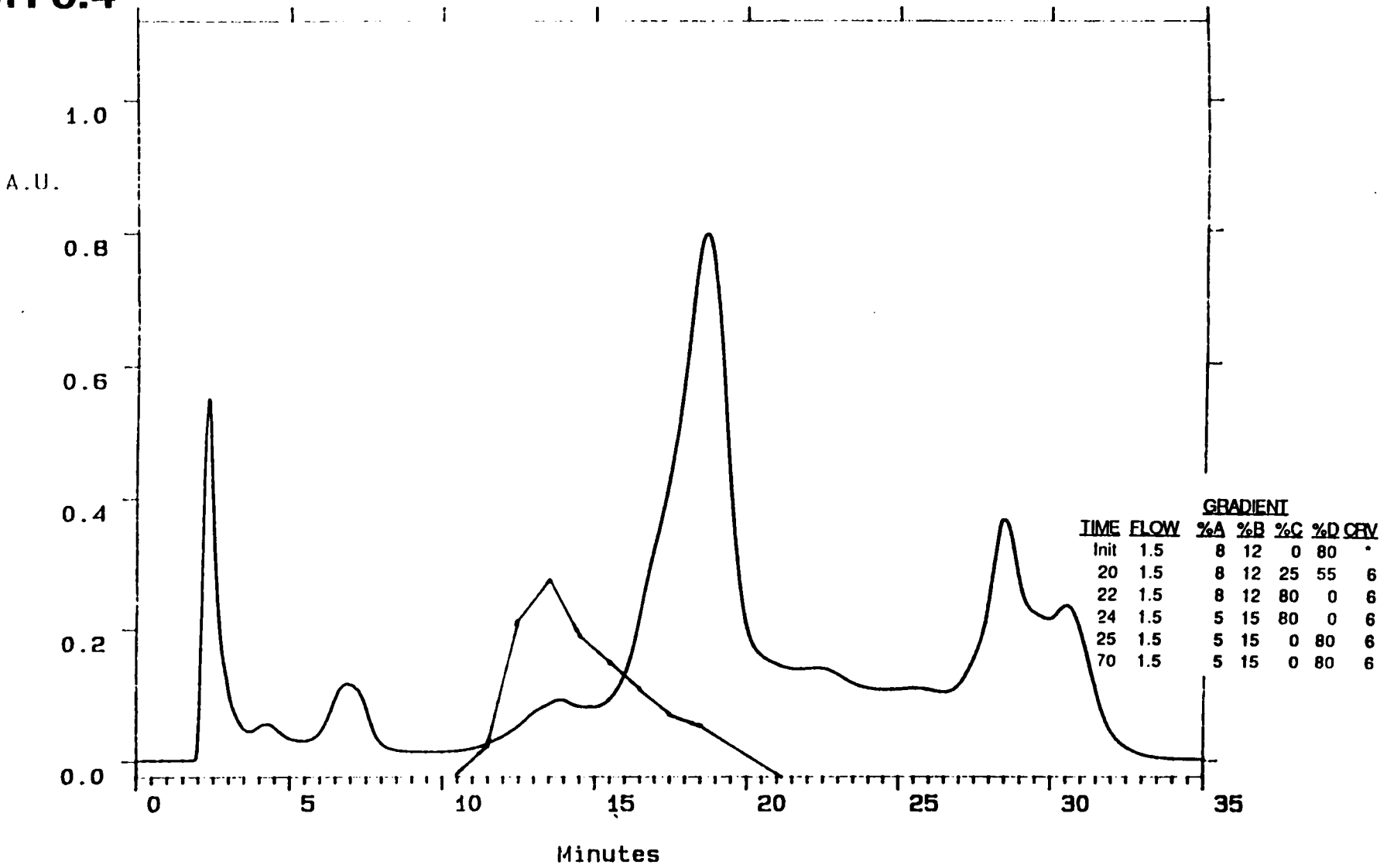
TIME	FLOW	%A	%B	%C	%D	CRV
Init	1.5	15	5	0	80	*
20	1.5	15	5	25	55	6
22	1.5	15	5	80	0	6
24	1.5	10	10	80	0	6
25	1.5	10	10	0	80	6
70	1.5	10	10	0	80	6

ACCELL™ QMA


pH 8.2
A.U.
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Minutes
GRADIENT
TIME FLOW %A %B %C %D CRV
Init 1.5 10 10 0 80 *
20 1.5 10 10 25 55 6
22 1.5 10 10 80 0 6
24 1.5 8 12 80 0 6
25 1.5 8 12 0 80 6
70 1.5 8 12 0 80 6

ACCELL™ QMA

pH 8.4

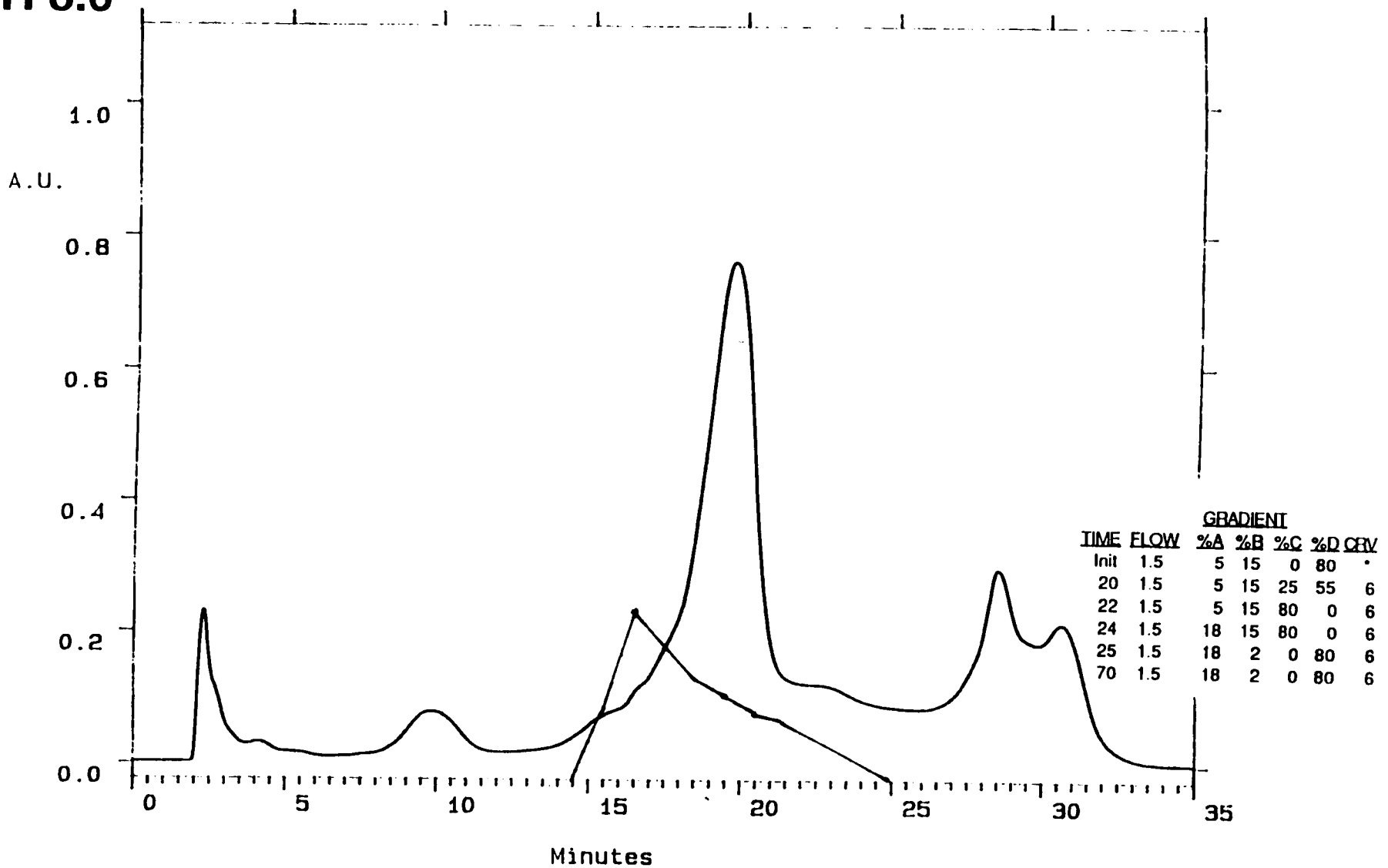


GRADIENT

TIME	FLOW	%A	%B	%C	%D	CRV
Init	1.5	8	12	0	80	*
20	1.5	8	12	25	55	6
22	1.5	8	12	80	0	6
24	1.5	5	15	80	0	6
25	1.5	5	15	0	80	6
70	1.5	5	15	0	80	6

ACCELL™ QMA

pH 8.6



GRADIENT

TIME	FLOW	%A	%B	%C	%D	CRV
Init	1.5	5	15	0	80	•
20	1.5	5	15	25	55	6
22	1.5	5	15	80	0	6
24	1.5	18	15	80	0	6
25	1.5	18	2	0	80	6
70	1.5	18	2	0	80	6

CONCLUSIONS

1. Adjustment of the pH of an ion-exchange separation of proteins produces dramatic changes in selectivity. Changes as small as 0.2 units can significantly improve the chromatography.

2. An automated system incorporating the Waters Auto-Blend™ Method permits the systematic optimization of the pH used for a protein in separation in a matter of hours.

3. Different chromatographic packing materials give different selectivity. The pH sensitivity of a particular purification depends on the column.

4. The DEAE 8HR and 40HR materials show identical selectivity on 8µ and 40µ packings, respectively.

5. Modern high resolution packing materials can be effectively used as the first step in a purification protocol. The yields are on the order of 90-100% with the DEAE 8HR, DEAE 40HR, and DEAE MemSep, and the degree of purification exceeds that commonly obtained with other techniques applied to crude extracts. These columns do not deteriorate rapidly with such samples and can be cleaned with aggressive regeneration procedures.