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Isotachophoresis on Polyacrylamide Gel

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Isotachophoresis on Polyacrylamide Gel

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Summary

Isotachophoresis, using Ampholine spacer ions, was applied to the fractionation of two multicomponent protein systems (serum and a urinary preparation tract with Hunter Factor activity) using polyacrylamide gel as a supporting medium. These studies were designed to determine whether isotachophoresis could provide higher load capacity than obtained in conventional electrophoresis or isoelectric focusing on polyacrylamide gel without loss of resolution. The effect of the Ampholine pI range, the relative and absolute concentrations of protein sample and Ampholine, of the ionic strength of the gel buffer, and of the stacking limits obtained in various gel buffers was investigated. It was found that Ampholine components can act as spacers, giving rise to bands in what otherwise would be a continuum of stacked protein zones. Loads greater by 1 to 2 orders of magnitude than in conventional polyacrylamide gel electrophoresis were applicable. The resotion obtained was greatly inferior to that in PAGE, suggesting that Ampholine does not provide a sufficient distribution of constituent mobilities under the employed conditions. Isotachophoresis was applied at the preparative scale in two-stage fractionations designed to resolve multicomponent systems (serum).

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INTRODUCTION

Zone electrophoresis in general, and polyacrylamide gel electrophoresis (PAGE) in particular, suffer from a relatively low load capacity, when applied to preparative-scale fractionation problems of the usual degree of difficulty, e.g., in the resolution of two species differing in mobility by 0.1 R_f units. Under these conditions, a load capacity of 1 to 2 mg/cm^2 of gel/component seems a realistic maximum (*1*). Isoelectric focusing in polyacrylamide gel (IFPA) permits use of higher sample loads (*2*). However, use of IFPA on a preparative scale has not been fully exploited to date—it is as yet limited to excision and elution or solubilization of gel slices (*2*, *3*). Preparative isoelectric focusing in sucrose gradients (*4*) is available but is subject to the problems related to protein precipitation at the pI. Against this background, Ornstein's prediction (*5*) of an unlimited load capacity for "steady-state stacking" (SSS) carries the greatest promise for preparative zone electrophoresis. For a protein of 60,000 mol wt in the Tris system of Ornstein and Davis (*5*, *6*) the "regulated" protein concentration within the stack is of the order of 200 mg/ml (*7*). This protein concentration can be increased further by altering the concentration of the stacking (BETA) phase buffer. Since the concentration of each protein component is fixed in SSS, an increase in load leads to an increase in zone width. Ornstein conceived of preparative SSS in which protein zones migrate electrophoretically into an elution chamber from which they are continuously (*8*) or intermittently (*1*) cleared. When high loads of protein components are stacked electrophoretically in successive broad zones and are successively eluted, homogeneous components should be collected at all elution times except during the elution of the boundaries between two adjacent stacked zones. [Note that both Ornstein's (*5*) and Jovin's (*7*) theory ignore the role of diffusion broadening of zone boundaries.] Although SSS is inherently the only fractionation method to provide increasing resolving power with increasing load, preparative SSS has not been successfully applied to date. Two innovations have recently provided fresh momentum for the application of SSS to preparative zone electrophoresis. The first is the availability of stacking at all pH values, 0° or 25°C, in the various multiphasic buffer systems generated on the basis of the Jovin theory (*9*). The second consists of the finding (*10*, *11*) that resolution in SSS can be improved by interposition of "spacer ions" between the stacked protein zones. To be effective in improving resolution, spacers should possess constituent

mobilities intermediate between those of each pair of adjacent proteins in the system. [Note that proteins within a stack are aligned in order of constituent mobilities (*5*, *7*).] The mixtures of polyamino–polycarboxylic acids available in various pI ranges under the trade name of Ampholine (LKB) have been used (*11*, *12*) previously and in this study as a source of spacer ions, and were assumed to provide a wide spectrum of constituent mobilities. Unfortunately, some confusion has been created by the fact that several authors (*10–15*) have designated both the original concept of SSS, and the new application in conjunction with spacer ions, by the single term *isotachophoresis*. In the interest of a clear discrimination between two different techniques we shall use isotachophoresis (ITP) to apply to SSS in the presence of spacer ions only while preserving SSS for zone electrophoresis in multiphasic (discontinuous) buffer systems (*5*, *7*, *9*).

Since at the present time, polyacrylamide (PA) is the most versatile and convenient matrix available in zone electrophoresis, ITP was applied in polyacrylamide gels and designated as ITPPA. The promise of high load capacity inherent in preparative ITPPA cannot be tested, however, prior to a systematic study of resolution in analytical ITPPA, since both high load capacity and high resolution are essential for fractionation. The present study, and a recent study by Griffith and Catsimpoolas (*12*) represent preliminary attempts to evaluate resolution in ITPPA as a function of several relevant parameters. Of particular interest was the question whether Ampholine was a suitable source of spacer ions for ITPPA, i.e., whether the constituent mobilities of the polyelectrolytes contained in Ampholine were intermediate between those of the fractionated proteins. Serum was chosen for our studies on ITPPA, since it seemed to provide a wide distribution of representative proteins for this purpose. The present study also tests the predicted high load capacity of ITPPA at the analytical and the preparative scale.

Since the constituent mobilities of proteins in gels are usually low compared to that of the buffer ions (CONSTITUENTS 1 and 2) used in multiphasic buffer systems (*9*), it is undesirable to retard the electrophoretic migration of protein by steric hindrance ("molecular sieving") if proteins are to migrate within the stacking limits (formed between buffer CONSTITUENTS 1 and 2) (*9*, *17*). Thus the maintenance of a stack in SSS or ITPPA depends on the selection of a gel with a "minimally restrictive pore size." [For other reasons, the same restriction holds for IFPA (*2*).] A minimally restrictive pore size may arbitrarily be defined as one that retards the migration of the macro-

molecule to the same degree as the migration of buffer ions or dye molecules. Open pore sizes are available in 1 to 5%C (*18*, *19*) PA gels of low, i.e., 2–5%T (*18*, *19*), such as have been used in IFPA (*2*). However, these low %T gels usually present severe problems of mechanical instability. This may account for the fact that preparative SSS has not been carried out to date. To improve mechanical stability, we have, therefore, applied ITPPA to gels of low %C, low %T gel, mechanically reinforced by the synchronous gelation of agarose with the polymerization of cross-linked acrylamide (*20*). An alternative method for formation of "large pore" gels is the 20–50%C gel recently described (*21*), but it has not yet been applied to ITPPA or IFPA. Methods for experimental determination of the maximal allowable gel concentration compatible with stacking of the protein(s) have been described previously (*19*, *22*, *23*).

In addition to the selection of a minimally restrictive pore size, ITPPA depends on the choice of a low enough "lower stacking limit"

TABLE 1

```
                        SYSTEM NUMBER
      INPUT DATA

DATE = 08/26/70     COMPUTER SYSTEM NUMBER = JCV-CHR     1958
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =    0  DEG. C.


          SPECIFIED CONSTITUENTS
CONSTITUENT 3 = NO.   99 , CHLORIDE -
CONSTITUENT 4 = NO.   99 , CHLORIDE -
CONSTITUENT 5 = NO.   99 , CHLORIDE -
CONSTITUENT 6 = NO.   97 , POTASSIUM +
CONSTITUENT 6 = NO.   83 , AMMONIA

          SPECIFIED CONCENTRATIONS
MAX CONC. ALLOWED    =    0.60
C1 IN PHASE ZETA(4) = 0.0400

          PHASE PI(9) AND PHASE ZETA(4)
PH(9) =    7.50          IONIC STRENGTH(9) =  0.015
UNSTACKING LIMITS(ALLOWED RANGE RM(1,9)) = -0.10 TO  -0.50
PH(4) = NOT SPECIFIED
STACKING LIMITS(MINIMAL ALLOWED RANGE)  RM(1,4) = -0.15 TO  RM(2,2) = -).20
MAX ABS(PH(2) - PH(4)) =  2.50
STACKING OPTIMIZATION = MINIMIZE ABS(RM(1,4))
SELECTION CONSTITUENT 2   IC12 =  1

          PHASE DELTA(10) - ELUTION BUFFER
RATIO IONIC STRENGTHS IS(10)/IS(9) =      3.0
MIN PH =       6.5
MAX PH =       8.5

          PHASE EPSILON(11) - LOWER BUFFER
IS =          0.05
PHI(6) =      0.80

          PHASE PSI(5) AND TAU(6) - RESTACKING PARAMETERS
RFMAX =  0.90
MAX ABS(PH(5) - PH(9)) =  2.00
```

(*continued*)

TABLE 1 (continued)

SYSTEM NUMBER

DATE = 08/26/70 COMPUTER SYSTEM NUMBER = JOV-CHB 1958
POLARITY = - (MIGRATION TOWARD ANODE) TEMPERATURE = 0 DEG. C.

CONSTITUENT 1 = NO. 23 , TES
CONSTITUENT 2 = NO. 82 , PHOSPHATE-DIBASIC
CONSTITUENT 3 = NO. 99 , CHLORIDE -
CONSTITUENT 6 = NO. 5 , 4-PICOLINE

	PHASES					
	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0400	0.0400		0.0583		
C2			0.0489		0.0575	
C3						0.1108
C6	0.0413	0.0413	0.0506	0.3075	0.3195	0.3476
THETA	1.032	1.032	1.033	5.278	5.556	3.138
PHI(1)	0.120	0.120		0.257		
PHI(2)			0.004		0.318	
PHI(3)						1.000
PHI(6)	0.116	0.116	0.972	0.049	0.237	0.319
RM(1)	-0.049	-0.049		-0.106		
RM(2)			-0.582		-0.704	
RM(3)						-1.626
RM(6)	0.082	0.082	0.690	0.035	0.168	0.226
PH	7.09	7.09	4.67	7.50	6.74	6.54
ION.STR.	0.0048	0.0048	0.0493	0.0150	0.0941	0.1108
SIGMA	0.517	0.517	6.132	1.621	10.813	24.972
KAPPA	132.	132.	1424.	396.	2416.	5500.
NU	-0.095	-0.095	-0.095	-0.065	-0.065	-0.065
BV	0.019	0.019	0.004	0.059	0.162	0.174

PHASE ETA(7) X1= 0.620 X2= 0.066 X3= 0.851 X4= 0.091

RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
TES	GM	9.17			5.34
1M PHOSPHORIC ACID	ML		19.58		
1N HCL	ML			44.31	
4-PICOLINE	GM	3.85	1.88	12.95	11.45
H2O TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		6.76	4.53	6.39	7.19
KAPPA(25 DEG.C.)		392.	2821.	10325.	1149.

(*continued*)

[RM(1,4)] such that the constituent mobility of the macromolecule in the stacking phase (ZETA) exceeds that of the trailing ion (CONSTITUENT 1) (*9*, *17*). Optimal selectivity of the multiphasic buffer

TABLE 1 (continued)

SYSTEM NUMBER

DATE = 08/26/70 COMPUTER SYSTEM NUMBER = JCV-CHR 1958

PHASE DELTA(10) - ELUTION BUFFER

IS = 0.045

0 DEG.C. PH	KAPPA	25 DEG.C. PH	KAPPA	C6	C4
6.50	2365.	6.35	4448.	0.1327	0.0450
7.00	2365.	6.85	4448.	0.3225	0.0450
7.50	2365.	7.35	4448.	0.9224	0.0450
8.00	2365.	7.85	4448.	2.8197	0.0450
8.50	2365.	8.35	4448.	8.8192	0.0450

PHASE EPSILON(11)-LOWER BUFFER

IS = 0.050

0 DEG.C. PH	KAPPA	25 DEG.C. PH	KAPPA	C6	C5
5.61	2615.	5.46	4912.	0.0625	0.0500

STACKING AND UNSTACKING RANGES

PHASE ZETA(4) OR PI(9)				PHASE BETA(2) OR LAMBDA(8)					PHASE GAMMA(3)			
RM(1)	PHI(1)	C(1)	C(6)	PH	RM(2)	PHI(2)	C(2)	C(6)	PH	C(3)	C(6)	PH
-0.050	0.122	0.0400	0.0433	7.10	-0.58	0.010	0.0487	0.0528	5.07	0.2331	0.2431	4.84
-0.068	0.167	0.0400	0.0818	7.26	-0.62	0.110	0.0448	0.0944	6.16	0.1710	0.2650	5.95
-0.086	0.210	0.0400	0.1336	7.38	-0.66	0.210	0.0419	0.1485	6.49	0.1360	0.3033	6.30
-0.104	0.254	0.0400	0.2043	7.49	-0.70	0.310	0.0396	0.2210	6.72	0.1124	0.3552	6.54
-0.123	0.301	0.0400	0.3035	7.59	-0.74	0.410	0.0378	0.3216	6.91	0.0948	0.4231	6.75
-0.145	0.353	0.0400	0.4486	7.70	-0.78	0.510	0.0363	0.4679	7.09	0.0807	0.5143	6.94
-0.170	0.414	0.0400	0.6747	7.81	-0.82	0.610	0.0350	0.6950	7.26	0.0689	0.6437	7.13
-0.200	0.488	0.0400	1.0658	7.94	-0.86	0.710	0.0339	1.0870	7.46	0.0584	0.8467	7.34
-0.240	0.585	0.0400	1.8823	8.11	-0.90	0.810	0.0330	1.9043	7.70	0.0487	1.2290	7.59
-0.300	0.733	0.0400	4.5421	8.40	-0.93	0.910	0.0322	4.5647	8.07	0.0389	2.3437	7.98

RESTACKING PARAMETERS

	PHASE PSI(5)						PHASE TAU(6)				
CT7	IS	RM(7)	PHI(7)	C(7)	C(6)	PH	C(7)	C(6)	PH	PHI(7)	KAPPA
24	0.014	-0.088	0.313	0.0450	0.2942	7.51	0.0309	0.0322	7.04	0.134	101.
25	0.007	-0.046	0.143	0.0494	0.2987	7.82	0.0339	0.0352	7.41	0.061	54.

(*continued*)

TABLE 1 (continued)

```
DATE = 02/29/72     COMPUTER SYSTEM NUMBER =  Chrambach29642
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =    0  DEG. C.


          SPECIFIED CONSTITUENTS
CONSTITUENT 1 = NO.   40 , GABA
CONSTITUENT 2 = NO.   32 , BICINE
CONSTITUENT 3 = NO.   99 , CHLORIDE -
CONSTITUENT 4 = NO.   99 , CHLORIDE -
CONSTITUENT 5 = NO.   99 , CHLORIDE -
CONSTITUENT 6 = NO.   38 , AMMEDIOL

          SPECIFIED CONCENTRATIONS
PHASE ALPHA(1) -    C1 =       0.04000        C6 =      0.01140
PHASE  BETA(2) -    C2 =       0.04390        C6 =      0.01530
PHASE GAMMA(3) -    C3 =       0.06630        C6 =      0.44740

          PHASE DELTA(10) - ELUTION BUFFER
RATIO IONIC STRENGTHS IS(10)/IS(9) =      3.0
MIN PH =      10.0
MAX PH =      11.0

          PHASE EPSILON(11) - LOWER BUFFER
IS =          0.05
PHI(6) =      0.80

          PHASE PSI(5) AND TAU(6) - RESTACKING PARAMETERS
RFMAX =  0.90
MAX ABS(PH(5) - PH(9)) =  2.00
```

(*continued*)

system is obtained if the stacking limits narrowly encompass the constituent mobility of the macromolecule. Thus one would select a more neutral buffer system for highly basic or highly acidic proteins, while extremes of pH would be suitable for ITPPA fractionation of relatively uncharged macromolecules. The multiphasic buffer systems output of Jovin's program (*9*) offers a wide choice of stacking limits both within a given system and between systems.

MATERIALS AND METHODS

1. The two protein preparations used were human serum and a urinary preparation of partially purified (stage 2) Hunter Correction Factor (*24*). The spacer ions used were Ampholine (LKB) with nominal pI ranges of 3–6, 3–10, 5–7, and 6–8. Ampholine with a pI range 3–8 was formed by 1:1 mixture of 3–6 and 6–8. Prior to isotachophoresis, Ampholine was neutralized by NaOH or, whenever possible, by the common constituent (CONSTITUENT 6) of the particular multiphasic buffer system used. The neutralized Ampholine was mixed and applied together with the protein sample.

TABLE 1 (continued)

1 SYSTEM NUMBER

DATE = 02/29/72 COMPUTER SYSTEM NUMBER = Chrambach 29642
POLARITY = - (MIGRATION TOWARD ANODE) TEMPERATURE = 0 DEG. C.

CONSTITUENT 1 = NO. 40 , GABA
CONSTITUENT 2 = NO. 32 , BICINE
CONSTITUENT 3 = NO. 99 , CHLORIDE -
CONSTITUENT 6 = NO. 38 , AMMEDIOL

	PHASES					
	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0400	0.0400		0.0470		
C2			0.0439		0.0516	
C3						0.0663
C6	0.0114	0.0114	0.0153	0.4281	0.4327	0.4474
THETA	0.285	0.285	0.349	9.100	8.379	6.748
PHI(1)	0.060	0.060		0.319		
PHI(2)			0.325		0.980	
PHI(3)						1.000
PHI(6)	0.210	0.210	0.932	0.035	0.117	0.148
RM(1)	-0.033	-0.033		-0.175		
RM(2)			-0.224		-0.676	
RM(3)						-1.626
RM(6)	0.090	0.090	0.401	0.015	0.050	0.064
PH	10.13	10.13	8.42	11.00	10.44	10.32
ION.STR.	0.0024	0.0024	0.0143	0.0150	0.0506	0.0663
SIGMA	0.227	0.227	1.541	1.418	5.472	13.154
KAPPA	59.	59.	378.	347.	1269.	3007.
MU	-0.145	-0.145	-0.145	-0.124	-0.124	-0.124
BV	0.010	0.010	0.024	0.057	0.105	0.130

RECIPES FOR BUFFERS OF PHASES ZETA(4), BETA(2), GAMMA(3), PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
GABA	GM	4.12			1.94
BICINE	GM		2.87		
1N HCL	ML			26.52	
AMMEDIOL	GM	1.20	0.64	18.82	18.01
H2O TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		9.36	7.95	9.59	10.24
KAPPA(25 DEG.C.)		136.	727.	5754.	802.

(*continued*)

2. Polyacrylamide gels of 3.5%T, 5%C (see Fig. 4) and 4–7.5%T, 3%C (see Fig. 1) were used. The polymerization of these gels was effected by 5.5 m*M* potassium persulfate (KP), 0.013 m*M* riboflavin (RN) and 6.54 m*M* *N*,*N*,*N*′,*N*′-tetramethylethylene diamine (TEMED) (final concentrations). The PA gels were prepared as pre-

TABLE 1 (coninued)

1 SYSTEM NUMBER

DATE = 02/29/72 COMPUTER SYSTEM NUMBER = Chranbach 29642

PHASE DELTA(10) - ELUTION BUFFER

IS = 0.045

0 DEG.C.		25 DEG.C.			
PH	KAPPA	PH	KAPPA	C6	C4
10.00	2081.	9.27	3999.	0.1689	0.0450
10.50	2081.	9.77	3999.	0.4368	0.0450
11.00	2081.	10.27	3999.	1.2841	0.0450

PHASE EPSILON(11)-LOWER BUFFER

IS = 0.050

0 DEG.C.		25 DEG.C.			
PH	KAPPA	PH	KAPPA	C6	C5
8.96	2301.	8.23	4417.	0.0625	0.0500

STACKING AND UNSTACKING RANGES

PHASE ZETA(4) OR PI(9)				PHASE BETA(2) OR LAMBDA(8)					PHASE GAMMA(3)			
RM(1)	PHI(1)	C(1)	C(6)	PH	RM(2)	PHI(2)	C(2)	C(6)	PH	C(3)	C(6)	PH
-0.005	0.010	0.0400	0.0006	9.33	-0.07	0.102	0.0439	0.0045	7.79	0.0	0.0	0.0
-0.033	0.060	0.0400	0.0114	10.14	-0.22	0.325	0.0439	0.0153	8.42	0.0	0.0	0.0
-0.060	0.110	0.0400	0.0364	10.42	-0.47	0.688	0.0439	0.0403	9.08	0.0	0.0	0.0
-0.088	0.160	0.0400	0.0782	10.61	-0.61	0.881	0.0439	0.0821	9.61	0.1321	0.2215	9.39
-0.115	0.210	0.0400	0.1399	10.75	-0.65	0.942	0.0439	0.1438	9.95	0.1006	0.2790	9.81
-0.143	0.260	0.0400	0.2255	10.88	-0.67	0.967	0.0439	0.2294	10.20	0.0813	0.3489	10.08
-0.170	0.310	0.0400	0.3404	10.98	-0.68	0.979	0.0439	0.3443	10.41	0.0682	0.4315	10.29
-0.198	0.360	0.0400	0.4913	11.08	-0.68	0.986	0.0439	0.4952	10.58	0.0587	0.5288	10.46
-0.225	0.410	0.0400	0.6873	11.17	-0.68	0.990	0.0439	0.6912	10.73	0.0515	0.6436	10.62
-0.253	0.460	0.0400	0.9412	11.26	-0.68	0.993	0.0439	0.9451	10.88	0.0459	0.7806	10.76
-0.280	0.510	0.0400	1.2704	11.35	-0.69	0.995	0.0439	1.2743	11.01	0.0414	0.9461	10.90
-0.308	0.560	0.0400	1.7008	11.43	-0.69	0.996	0.0439	1.7047	11.14	0.0377	1.1498	11.03
-0.335	0.610	0.0400	2.2712	11.52	-0.69	0.997	0.0439	2.2751	11.27	0.0346	1.4062	11.16
-0.363	0.660	0.0400	3.0434	11.62	-0.69	0.998	0.0439	3.0473	11.40	0.0320	1.7385	11.29
-0.390	0.710	0.0400	4.1218	11.72	-0.69	0.998	0.0439	4.1257	11.53	0.0298	2.1856	11.42

RESTACKING PARAMETERS

PHASE PSI(5)						PHASE TAU(6)				
CT7	IS	RM(7)	PHI(7)	C(7)	C(6)	PH	C(7)	C(6)	PH	PHI(7)	KAPPA

(continued)

TABLE 1 (continued)

```
DATE = 03/02/72     COMPUTER SYSTEM NUMBER =  Chrambach29643
POLARITY = - (MIGRATION TOWARD ANODE)      TEMPERATURE =    0  DEG. C.


          SPECIFIED CONSTITUENTS
CONSTITUENT 1 = NO.  40 , GABA
CONSTITUENT 2 = NO.  32 , BICINE
CONSTITUENT 3 = NO.  99 , CHLORIDE -
CONSTITUENT 4 = NO.  99 , CHLORIDE -
CONSTITUENT 5 = NO.  99 , CHLORIDE -
CONSTITUENT 6 = NO.  38 , AMMEDIOL

          SPECIFIED CONCENTRATIONS
PHASE ALPHA(1) -   C1 =      0.04000        C6 =      0.03640
PHASE  BETA(2) -   C2 =      0.04390        C6 =      0.04030
PHASE GAMMA(3) -   C3 =      0.06630        C6 =      0.44740

          PHASE DELTA(10) - ELUTION BUFFER
RATIO IONIC STRENGTHS IS(10)/IS(9) =      3.0
MIN PH =      10.0
MAX PH =      11.0

          PHASE EPSILON(11) - LOWER BUFFER
IS =          0.05
PHI(6) =      0.80

          PHASE PSI(5) AND TAU(6) - RESTACKING PARAMETERS
RFMAX =  0.90
MAX ABS(PH(5) - PH(9)) =  2.00
```

(*continued*)

viously described (*19*). Analytical gels were 6 mm in diameter and 42 mm in length.

In all other cases, agarose–PA gels containing 2% acrylamide, 0.2% *N,N'*-methylenebisacrylamide (Bis), and 0.25% agarose were prepared. The procedure (*25*) of the polymerization of acrylamide simultaneously with the gelation of agarose is a modification of the procedure of Peacock and Dingman (*20*). By using the polymerization conditions of PAGE (*19*), which give rise to polymerization within 10 min, the temperature of an agarose–acrylamide–Bis mixture was lowered concomitantly with free radical polymerization to achieve a synchronous gelation of agarose and cross-linked acrylamide. Details are given in Appendix I.

All gels contained upper gel (stacking gel, BETA phase) buffer of one of the following alternative multiphasic buffer systems: systems B (*19*), 1958, 2964.2, and 2964.3 (*9*). The composition of these systems is described in Table 1. The various systems were used either at the ionic strength stated in the systems output (*9*) or at a multiple (designated as $2\times$, $3\times$, . . ., $n\times$) of this ionic strength. Gels were stained

TABLE 1 (continued)

I SYSTEM NUMBER

DATE = 03/02/72 COMPUTER SYSTEM NUMBER = Chrambach 20643
POLARITY = - (MIGRATION TOWARD ANODE) TEMPERATURE = 0 DEG. C.

CONSTITUENT 1 = NO. 40 , GABA
CONSTITUENT 2 = NO. 32 , BICINE
CONSTITUENT 3 = NO. 99 , CHLORIDE -
CONSTITUENT 6 = NO. 38 , AMMEDIOL

	PHASES					
	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0400	0.0400		0.0470		
C2			0.0439		0.0516	
C3						0.0663
C6	0.0364	0.0364	0.0403	0.4281	0.4327	0.4474
THETA	0.910	0.910	0.918	9.100	8.379	6.748
PHI(1)	0.110	0.110		0.319		
PHI(2)			0.688		0.980	
PHI(3)						1.000
PHI(6)	0.121	0.121	0.750	0.035	0.117	0.148
RM(1)	-0.060	-0.060		-0.175		
RM(2)			-0.475		-0.676	
RM(3)						-1.626
RM(6)	0.052	0.052	0.322	0.015	0.050	0.064
PH	10.42	10.42	9.08	11.00	10.44	10.32
ION.STR.	0.0044	0.0044	0.0302	0.0150	0.0506	0.0663
SIGMA	0.416	0.416	3.265	1.418	5.472	13.154
KAPPA	106.	106.	775.	347.	1269.	3007.
MU	-0.145	-0.145	-0.145	-0.124	-0.124	-0.124
BV	0.018	0.018	0.039	0.057	0.105	0.130

RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
GABA	GM	4.12			1.94
BICINE	GM		2.87		
1N HCL	ML			26.52	
AMMEDIOL	GM	3.83	1.69	18.82	18.01
H20 TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		9.65	8.51	9.59	10.24
KAPPA(25 DEG.C.)		247.	1417.	5754.	302.

(*continued*)

by the procedure of Awdeh (*26*) or sliced and analyzed for pH as described previously (*2, 3, 22, 23*).

3. Preparative ITPPA was carried out in the redesigned fractophorator apparatus as described previously (*4*) except as follows. For use with an agarose–acrylamide gel, a 2-mm thick porous Teflon plate

TABLE 1 (continued)

```
1                    SYSTEM NUMBER

DATE = 03/02/72     COMPUTER SYSTEM NUMBER =  Chrambach 2065

              PHASE DELTA(10) - ELUTION BUFFER
IS = 0.045
      0 DEG.C.              25 DEG.C.
    PH        KAPPA      PH        KAPPA       C6         C4
  10.00       2081.    9.27       3999.    0.1669    0.0450
  10.50       2081.    9.77       3999.    0.4366    0.0450
  11.00       2081.   10.27       3999.    1.2841    0.0450

              PHASE EPSILON(11)-LOWER BUFFER
IS = 0.050
      0 DEG.C.              25 DEG.C.
    PH        KAPPA      PH        KAPPA       C6         C5
   8.96       2301.    8.23       4417.    0.0025    0.0500

              STACKING AND UNSTACKING RANGES
PHASE ZETA(4) OR PI(9)              PHASE BETA(2) OR LAMBDA(8)        PHASE GAMMA(3)
 RM(1) PHI(1)  C(1)    C(6)    PH  RM(2) PHI(2)  C(2)    C(6)    PH    0(3)    0(6)    PH
-0.005 0.010 0.0400 0.0006  9.33 -0.07 0.102 0.0439 0.0015  7.79 0.0     0.0     0.0
-0.033 0.060 0.0400 0.0114 10.14 -0.22 0.325 0.0439 0.0135  8.42 0.0     0.0     0.0
-0.060 0.110 0.0400 0.0364 10.42 -0.47 0.688 0.0439 0.0403  9.08 0.0     0.0     0.0
-0.088 0.160 0.0400 0.0782 10.61 -0.61 0.881 0.0439 0.0821  9.61 0.1321  0.2215  0.59
-0.115 0.210 0.0400 0.1309 10.75 -0.65 0.942 0.0439 0.1438  9.95 0.1006  0.2790  0.81
-0.143 0.260 0.0400 0.2255 10.88 -0.67 0.967 0.0439 0.2294 10.20 0.0813  0.3489 10.08
-0.170 0.310 0.0400 0.3404 10.98 -0.68 0.979 0.0439 0.3483 10.41 0.0682  0.4315 10.20
-0.198 0.360 0.0400 0.4913 11.08 -0.68 0.986 0.0439 0.4952 10.53 0.0587  0.5288 10.46
-0.225 0.410 0.0400 0.6873 11.17 -0.68 0.990 0.0439 0.6912 10.73 0.0515  0.6436 10.62
-0.253 0.460 0.0400 0.9412 11.26 -0.68 0.993 0.0439 0.9451 10.88 0.0459  0.7806 10.76
-0.280 0.510 0.0400 1.2704 11.35 -0.69 0.995 0.0439 1.2743 11.01 0.0414  0.9461 10.90
-0.308 0.560 0.0400 1.7008 11.43 -0.69 0.996 0.0439 1.7047 11.14 0.0377  1.1498 11.03
-0.335 0.610 0.0400 2.2712 11.52 -0.69 0.997 0.0439 2.2751 11.27 0.0346  1.4062 11.16
-0.363 0.660 0.0400 3.0434 11.62 -0.69 0.998 0.0439 3.0473 11.40 0.0320  1.7385 11.29
-0.390 0.710 0.0400 4.1218 11.72 -0.69 0.998 0.0439 4.1257 11.53 0.0298  2.1856 11.42

              RESTACKING PARAMETERS
    PHASE PSI(5)                                    PHASE TAU(6)
CT7  IS   RM(7)  PHI(7) C(7)    C(6)    PH          C(7)    C(6)    PH  PHI(7) KAPPA
          NO CONSTITUENT FOUND
```

was inserted into the bottom of the gel column. Prior to insertion, the plate was immersed in a suction flask and deaerated by aspiration. The column, sealed with Parafilm, was immersed in water contained in a jacketed beaker at room temperature. The polymerization mixture was pipetted into the column and overlayered by water at 45°C. The flow of 0°C coolant into the jacketed beaker and illumination were then initiated simultaneously. After 40 min, the Parafilm seal was removed from the bottom of the column which was then inserted into the lower buffer reservoir of the apparatus maintained at 0°C. A load of 200 mg serum, 200 μl 50% sucrose, and 300 μl, pI 6–8, Ampholine was applied. Electrophoresis was conducted at 6 mA. Fractions of 1.3-ml elution buffer were collected at 10 min intervals. Elution buffer (system B) consisted of lower gel buffer at its final concentration (LGB × 1) prior to the elution of the front and 0.3624 M Tris, 0.0168 M HCl (pH 9.45), in 25% sucrose, subsequent to the elution of the front.

4. Preparative ITPPA was also carried out with the Polyprep 100 apparatus (Buchler Instruments Div.) as described previously (Appendix III of Ref. *4*) except that only a stacking gel was used and the sample was applied with Ampholine.

In the fractionation of urinary Hunter Corrective Factor, the gel was supported by a nylon mesh disc (available from Buchler Instruments). ITPPA was carried out in system 1958 in a 70-ml gel of 3.5%T, 2.5%C. pI 5–7 Ampholine (3.7 ml + 1.3 ml water, titrated to pH 7.0 with NaOH) was mixed with a 15-ml sample containing 8.4 mg protein/ml, 78 activity units/mg protein, 20% (w/v) sucrose, and 0.003% (w/v) bromphenol blue. Electrophoresis was performed at 20 mA; elution buffer flow rate was 0.8 ml/min.

For the fractionation of serum, system 2964.3 was used. A 50-ml stacking gel was made at a concentration of 3.5%T, 5%C with 200 μl TEMED/100 ml. Upper buffer was circulated at 0.3 ml/min from a thermostated reservoir (500 ml); lower buffer was circulated at 0.3 ml/min at room temperature. The load was 300 mg serum protein, 1.5 ml Ampholine (a 1:1 mixture of pI ranges 6–8 and 8–10). The elution buffer consisted of 1.284 *M* ammediol (2-amino-2-methyl-1,3-propanediol) in 25% sucrose brought to pH 10.24 by 1 *N* HCl, pumped at 0.8 ml/min. Electrophoresis was conducted at 25 mA/10.6 cm².

RESULTS

Determination of a "Minimally Restrictive" Pore Size for Serum Proteins

As pointed out above, ITPPA requires a relatively nonrestrictive pore size. Stacking in PAGE is used as a criterion of nonrestrictiveness of the gel. A stacking gel (system 2964.3, 3%C) at 4, 5, 6, and 7.5%T produced the patterns of serum shown in Fig. 1A. In a 4%T, 3%C gel, the stainable protein components of serum are stacked, whereas at higher gel concentrations, higher proportions of serum protein components are unstacked. Accordingly, a 4%T, 3%C gel represents a minimally restrictive pore size.

A stacking gel (system B) of serum proteins in the PA–agarose gel specified above is shown on Fig. 1B. Serum protein loads (from left to right) were 25, 50, 75, 100, and 150 μg/gel. Under these conditions, stacking of serum proteins appears not as complete as at pH 10.42, but it seems adequate for most of the serum protein components.

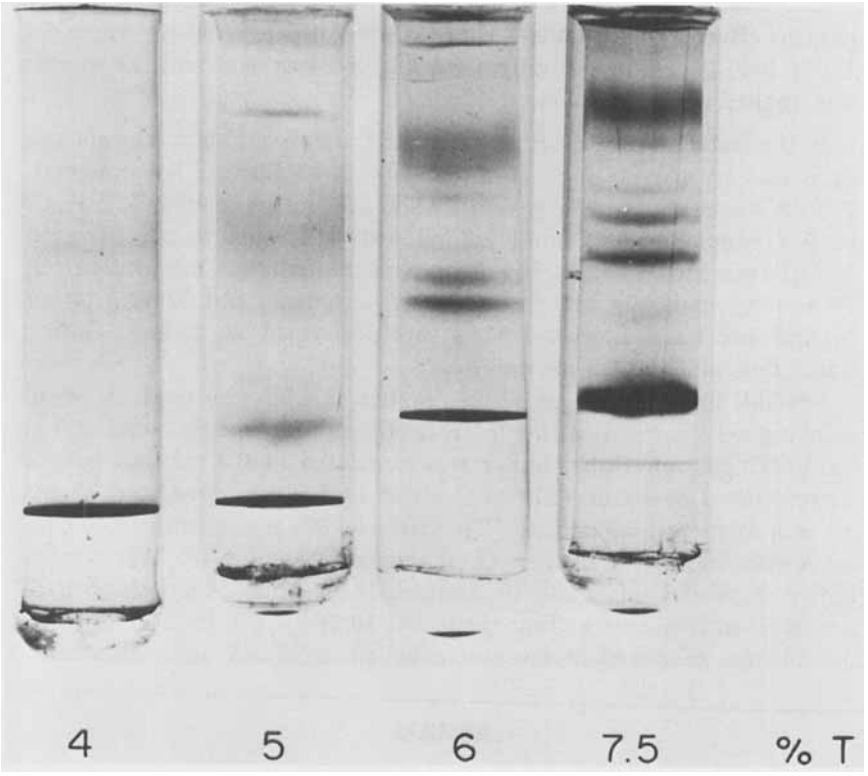

FIG. 1 A. Serum fractionations by ITPPA; see Table 1 for experimental details.

Demonstration of ITP in Polyacrylamide Gel

Figure 2 demonstrates an ITPPA fractionation of human serum at pH 9.65, 0°C (system B, phase BETA × 2, 2.2%T, 9%C, 0.25% agarose). Ampholine (pI 6–8) load on the gel varied from 40 (A) to 100 (C) μl. Alternate gels were stained or sliced and analyzed for pH. The pH-distance profiles of these gels showed that a stack is formed, maintained, and traverses these gels irrespective of Ampholine load. The point of inflection on the pH profiles between the (upper) ZETA phase and the (lower) BETA phase designates the position of the stack (indicated by the dotted lines in Fig. 2). In addition to this evidence of stacking, positive demonstration of ITP would require that the protein

and ampholyte zones within the stack be interspersed. The stained regions of the gels in Fig. 2 indicate the positions of the serum proteins: these are nonuniformly distributed between the center and leading edge of the stained zone that is presumably coextensive with the stack. The position of Ampholine components was inferred from the effect of increasing Ampholine concentration on the width of the stacked protein zone. The width of the protein zone increases with increasing load of Ampholine, suggesting that at least part of the Ampholine components acted as spacer ions. Thus, ITPPA with Ampholine spacers can be made operative in polyacrylamide gel.

Significantly, the pH values (ZETA and BETA) were unaffected by the presence of up to 100 μl Ampholine in the gel. However, under different conditions of Ampholine pI range and buffer system, it may be necessary to increase the ionic strength of the buffer or decrease the Ampholine load in order to avoid perturbation of the buffer pH by the Ampholine.

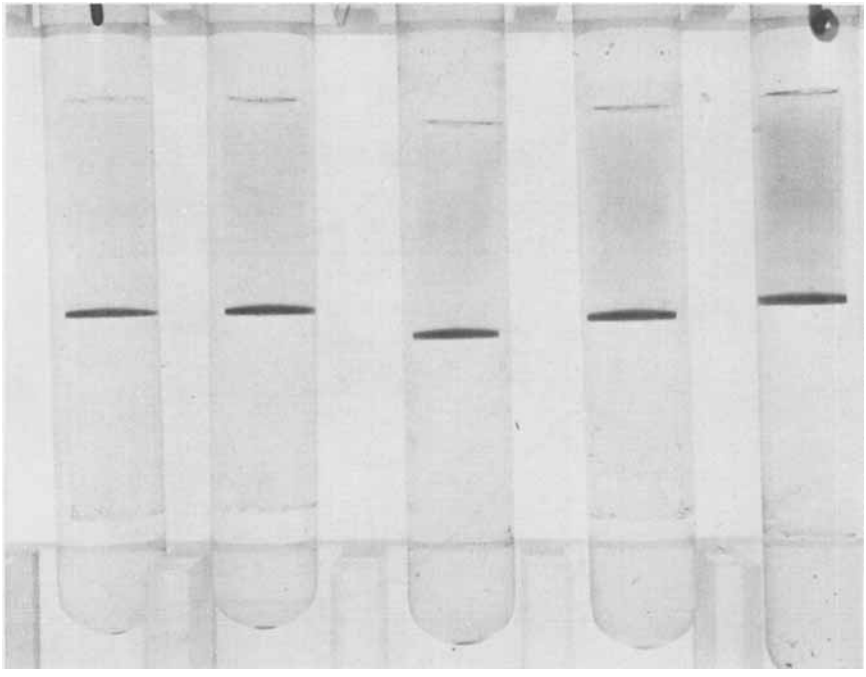

FIG. 1B. Serum fractionations by ITPPA; see Table 1 for experimental details.

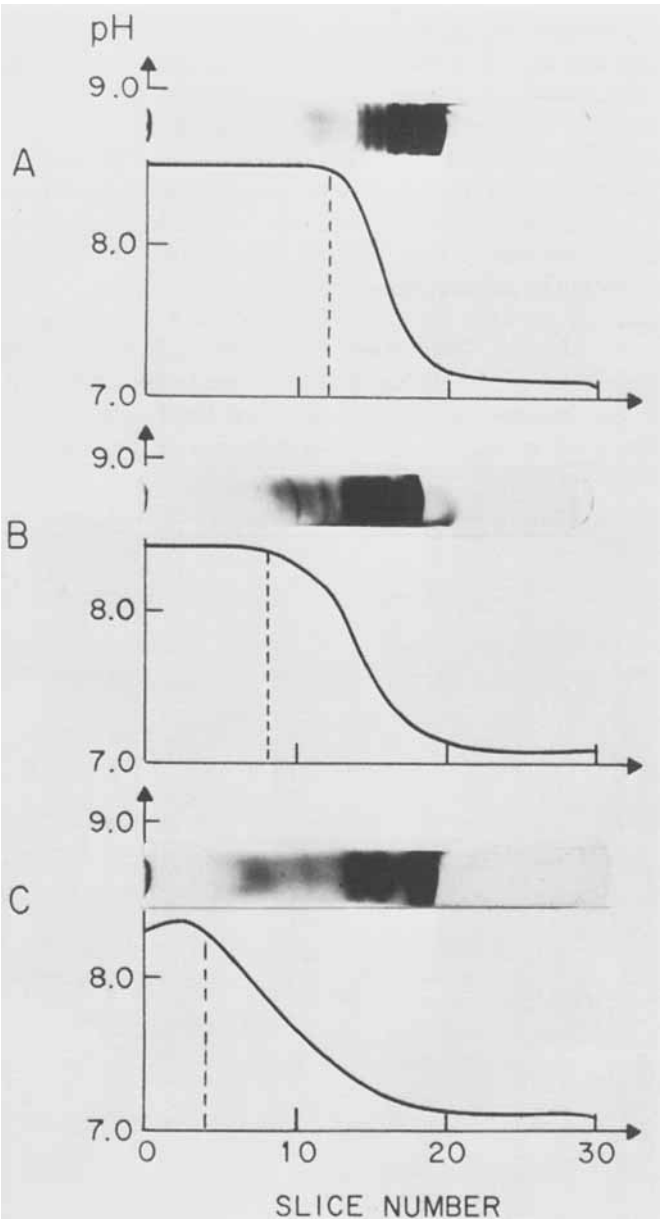

FIG. 2. Serum fractionations by ITPPA; see Table 1 for experimental details.

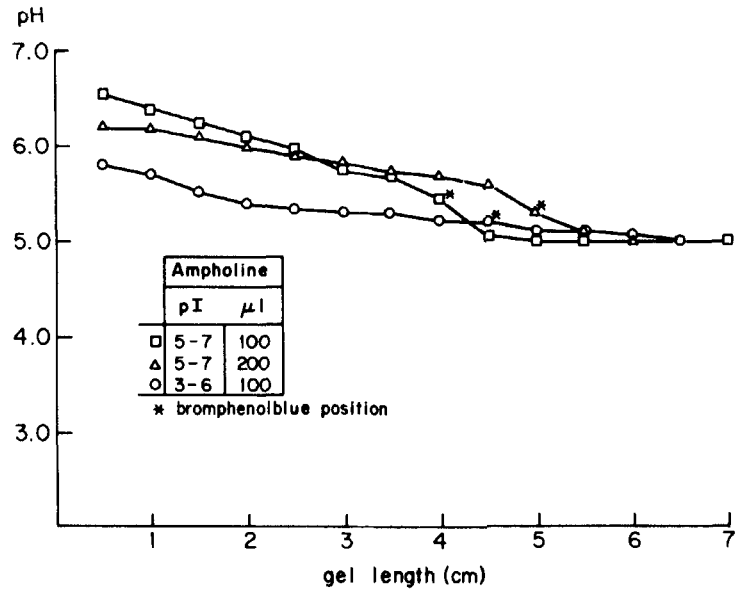

FIG. 3. Hunter Correction Factor fractionation by ITPPA; see Table 1 for experimental details.

Figure 3 shows the effect of spacer ions on the positions of stacked components in a different way. Here, the position of bromphenol blue (in the stack) is measured as a function of load and pI range of the Ampholine when other conditions are held constant (time, voltage gradient, current density). Lowering the pI range of the spacers (presumably increasing their average mobilities) results in an increase in the velocity of bromphenol blue. Increasing the Ampholine load has the same effect. Since the net charge and mobility of the dye are independent of pH in this range, this is not likely to be due to an effect of gel pH on the dye. Instead increase in dye mobility probably arises from the fact that the composition or concentration of Ampholine is altered in the zones adjacent to that of the dye.

Variation of Ampholine Load

Figure 4 shows the effect of Ampholine load on resolution. Fractionation was carried out at pH 10.13 (system 2964.2, phase BETA, 3.5%T, 5%C, Ampholine pI range 7–10). Serum protein load was constant at 1500 μg/gel. Some minimal resolution of the serum proteins is obtained

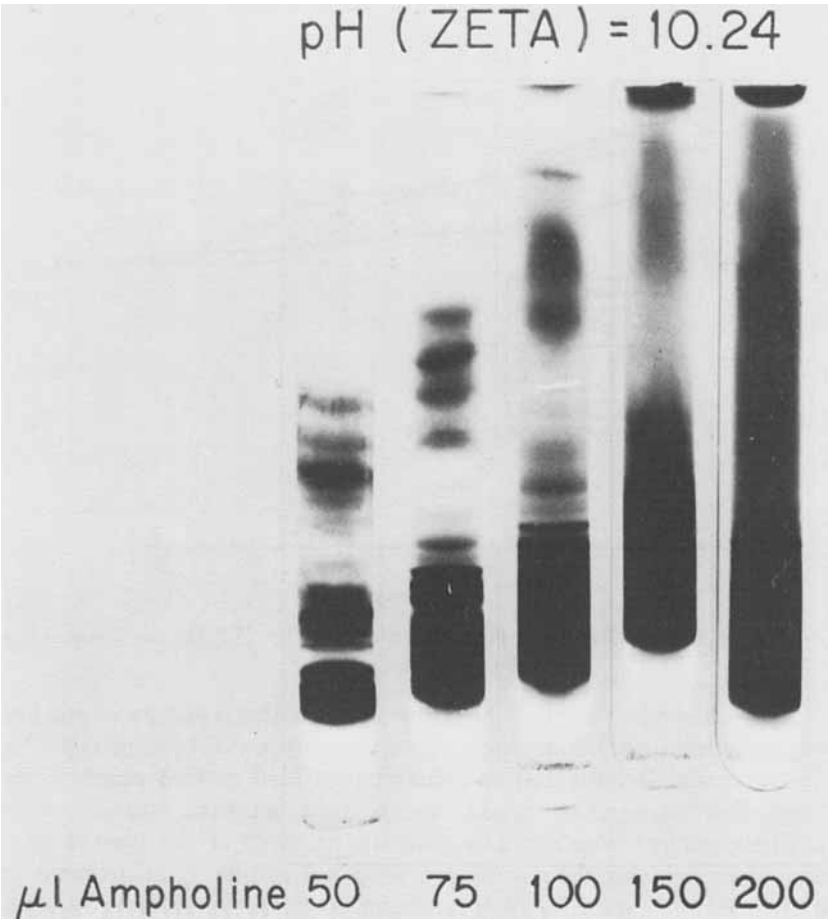

FIG. 4. Serum fractionation by ITPPA; see Table 1 for experimental details.

with Ampholine loads around 50 µl/gel. Figure 5C shows that at constant protein load and ionic strength and at very low Ampholine concentration, no resolution is obtained, since all proteins are stacked. At high Ampholine concentration, resolution is also poor, since protein components of the stack are fused. Resolution is optimal at intermediate Ampholine loads.

Variation of Ionic Strength

Figure 5 shows the effect of ionic strength on fractionation of 1000 µg of serum by analytical ITPPA (system B, phase BETA, 2.2%T,

9%C, 0.25% agarose). Ampholine (pI range 6–8) load was variable at constant protein load. Fractionations were carried out at 1× (Fig. 5A), 2× (Fig. 5B and C), and 4× (Fig. 5D) ionic strength. At any of the levels of ionic strength, the zone of stainable protein increased in width with increasing Ampholine load. Resolution is distinctly better at 2× compared to 1× or 4× ionic strength (Fig. 5B and C).

Variation of Protein Load

Analytical ITPPA gels (system B, phase BETA, 2.2%T, 9%C) at constant ionic strength, 1× (Fig. 6B) and 4× (Fig. 6A), were loaded with a constant amount of either 100 μl, pI 6–8, Ampholine (Fig. 6A) or 200 μl, pI 3–10, Ampholine (Fig. 6B). Serum load on these gels was varied between 0.5 and 45 mg/gel. At constant Ampholine load the

TABLE 2

Serum Fractionations by ITPPA at the Analytical Scale (Comparison of Data from Figs. 1 to 7)

Figure No.	Buffer system	Protein (μg)	Ampholine (μl)	Ampholine (pI range)	*I* (ZETA)	%T	%C	Agarose (%)
1A	2964.3	200	0		1X	4 5 6 7.5	3	0
1B	B	140	0	6–8	1X	2.2	9	0
2A	B	1000	40	6–8	2X	2.2	9	0.25
2B			60					
2C			100					
3	1958	1000[a]	100	5–7	1X	3.5	2.5	0
			200	5–7				
			100	3–6				
4	2964.2	1500	50–200	7–10	1X	3.5	5	0
5A	B	1000	10–200	3–8	1X	2.2	9	0.25
5B			10–40	6–8	2X			
5C			40–100	6–8	2X			
5D			50–400	6–8	4X			
6A	B	50–5000	100	6–8	4X			
6B		5000–45000	200	3–10	1X			
7		100–5000	40–2000	6–8	1X			

[a] Hunter Correction Factor preparation.

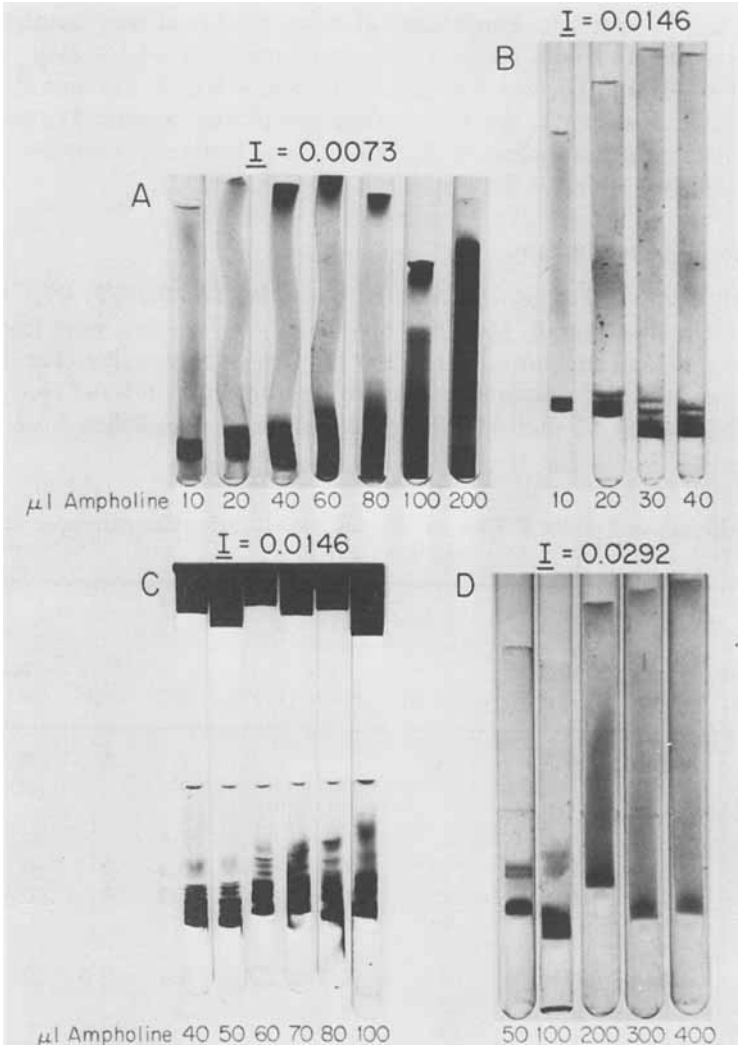

FIG. 5. Serum fractionations by ITPPA; see Table 1 for experimental details.

width of the stack increases in proportion to load (Fig. 6B) under the conditions used. In the range of 0.5 to 5.0 mg, resolution appears to improve in proportion to load (Fig. 6A). Migration of components with low constituent mobilities into the gel also appears improved in proportion to protein load (Fig. 6A).

When urinary Hunter Correction Factor was loaded onto analytical ITPPA gels (system 1958, 3.5%T, 2.5%C) at levels of about 2500 and 5000 μg, wall separation resulted in the upper one-third of the gel. This effect appears to be related to load and may be due to protein precipitation and high resistivity at the gel surface (ohmic heating) coupled with the mechanical instability of the gel.

Variation of Load at a Constant Protein–Ampholine Load Ratio

Serum was fractionated by ITPPA (system B, 2.2%T, 9%C, 0.25% agarose, Ampholine pI range 6–8) using variable ratios of milligrams protein per microliters Ampholine: 0.1/40, 0.5/200, 1.0/400, 2.0/800, and 5.0/2,000 (Fig. 7). At a constant protein–Ampholine ratio, the fractionation patterns depend on total load applied. A spacer effect appears abruptly between 40 and 200 μl Ampholine load. The separation between an acidic protein fraction (rapid migration) and a (pre-

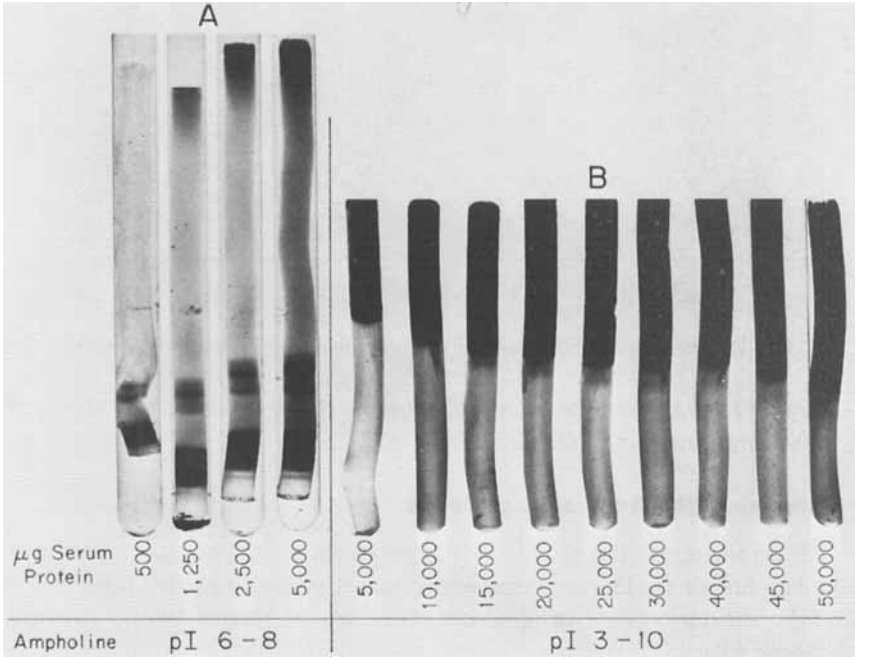

FIG. 6. Serum fractionations by ITPPA; for see Table 1 for experimental details.

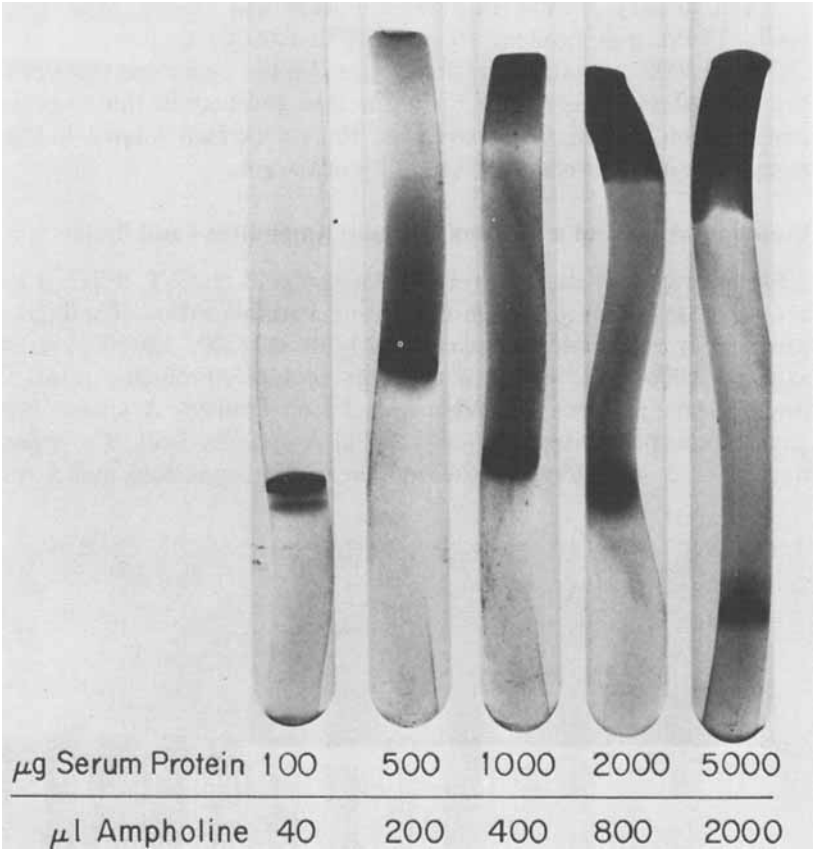

FIG. 7. Serum fractionation by ITPPA; see Table 1 for experimental details.

sumably) relatively basic group of proteins (slower migration) improves with increasing Ampholine load.

Variation of the Ampholine pI Range

Since the mobilities of neither the protein nor the various ampholytes in Ampholine are known, selection of spacer ions was made on the basis of the rationale that Ampholines with high pI ranges should provide spacers with low, average constituent mobilities in electrophoretic migration toward the anode. Therefore, since frequently protein mobilities are low compared to buffer ions and presumably to Ampholine

constituents, high pI ranges should be selected for the fractionation of anionic proteins in ITPPA. The overlap between proteins and ampholytes can then be tested as shown in the experiment depicted in Fig. 1. The protein zone is stained. The ampholyte effect as "spacer ions" is inferred from the relative width of the stained protein zone and from the relative width of the corresponding sigmoidal curve of distance vs pH on the gel. Alternatively, the position of Ampholine can be detected directly by staining methods (*12*, *27*).

Comparison of the serum patterns between Fig. 6A and B, gels 4 and 5 (from left to right) shows the superiority, under the conditions used, of the more basic pI 6–8 Ampholine over that with a pI 3–10. Presumably, resolution in gel 4 is aided by the larger fraction of relatively slowly migrating ampholytes in that preparation.

Preparative ITPPA

a. Preparative ITPPA (system 1958, pI range 5–7, Ampholine, 3.5%T, 2.5%C) of urinary Hunter Correction Factor (*24*) was carried out on 230 mg of protein, using the Polyprep 100 apparatus. Recovery of activity was 70%, but purification achieved was twofold only.

b. The following are two procedures for preparative ITPPA, followed by analytical PAGE fractionation of the eluate (ITPPA-PAGE).

1. Figure 8 depicts the elution profile (absorbance at 280 nm) of a preparative ITPPA (system B, 2.2%T, 9%C, and 0.25% agarose) fractionation carried out in the redesigned fractophorator apparatus on 200 mg serum protein and 0.3 ml, 40%, pI 6–8, Ampholine. Eluate fractions 18, 35, 43, and 55 under the peaks were subjected to analytical PAGE (system B, 4, 5, 6 and 7.5%T, 3%C), with the resulting K_R and Y_0 values shown in Table 3. The number of serum components detected on the basis of K_R-Y_0 pairs in the limited part of the eluate analyzed is 11. The characteristic K_R-Y_0 pairs for each component differ from those found in the same buffer system in two-stage fractionations with PAGE or pore gradient electrophoresis (P-G-E) as the first stage (*16*).

2. Preparative ITPPA (system 2964.3, 3.5%T, 5%C) was carried out on 300 mg of serum protein and 0.6 ml, 40%, pI 6–8 Ampholine in a stacking gel using the Polyprep 100 aparatus. It was followed by PAGE (system B, 4, 5, 6 and 7.5%T, 3%C) analysis of the eluate frac-

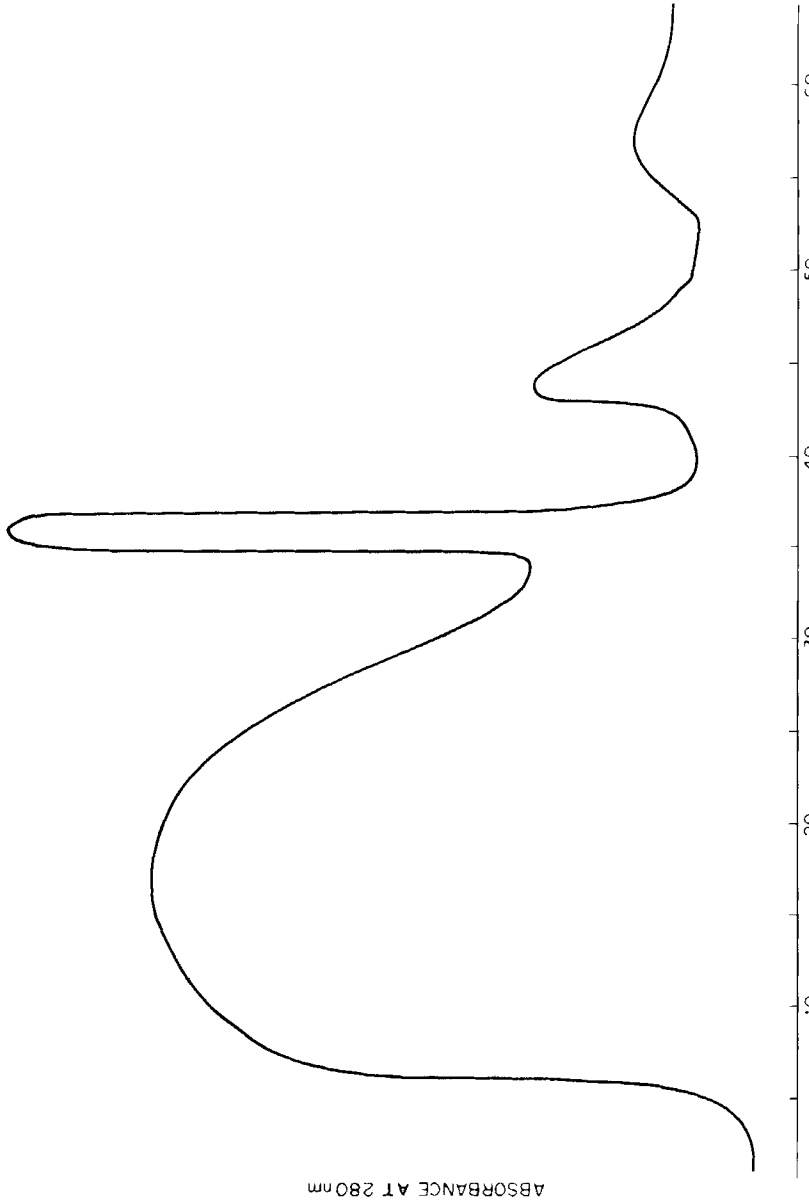

FIG. 8. Elution profile of a ITPPA-PAGE fractionation of serum. Maximal peak height is approximately 1 O.D. (280 nm) unit. ITPPA is carried out on the redesigned fractophorator apparatus. Load—200 mg of serum protein; current—6 mA; fraction time—10 min.

TABLE 3

PAGE (System B, 3%C) Analysis of Eluate Fractions from Preparative ITPPA (System B, 3.5%T, 5%C)

	Eluate fraction No.							
	18		35		43		55	
No.	K_R	Y_0	K_R	Y_0	K_R	Y_0	K_R	Y_0
1	0.310	5.20	0.354	5.81	0.301	3.41	0.256	2.76
2	0.186	1.65	0.265	3.18	0.287	3.99	0.217	3.02
3	0.188	2.77	0.259	4.02	0.239	3.54	0.144	1.73
4	0.144	1.72	0.210	2.91	0.176	2.02	0.128	1.72
5	0.088	1.17	0.156	1.84	0.153	2.10	0.083	1.18
6	0.079	1.14	0.119	1.52	0.114	1.51	0.083	1.53
7	0.079	1.30	0.102	1.53	0.086	1.21	0.078	1.60
8	0.068	1.24	0.081	1.57	0.082	1.47	0.078	1.88
9	0.070	1.38	0.092	1.76	0.082	1.78		
10	0.067	1.44	0.087	1.87				
11	0.070	1.80	0.079	1.96				

tions with the results depicted in Fig. 9 (left column). It appears that the number of protein components of serum recognized by R_f appears higher than that found in a two-stage fractionation of preparative P-G-E and analytical PAGE (right column) (*16*). Also, the nature of the eluate fractions from ITPPA differs from that in P-G-E or PAGE in that late and early eluate fractions alike contain proteins within the whole spectrum of R_f values.

DISCUSSION

Comparison of Methods for Fractionation on the Basis of Molecular Net Charge

Electrophoretic resolution between molecules of varying molecular size is most efficient in a molecular sieve of a specific, optimal pore size (*28*, *29*). Such fractionations are, however, restricted with regard to load capacity to about 1 mg protein/component/cm^2 of gel (assuming fractionation between two bands of 0.1 R_f difference). High load capacity is, at present, at least potentially associated with two methods

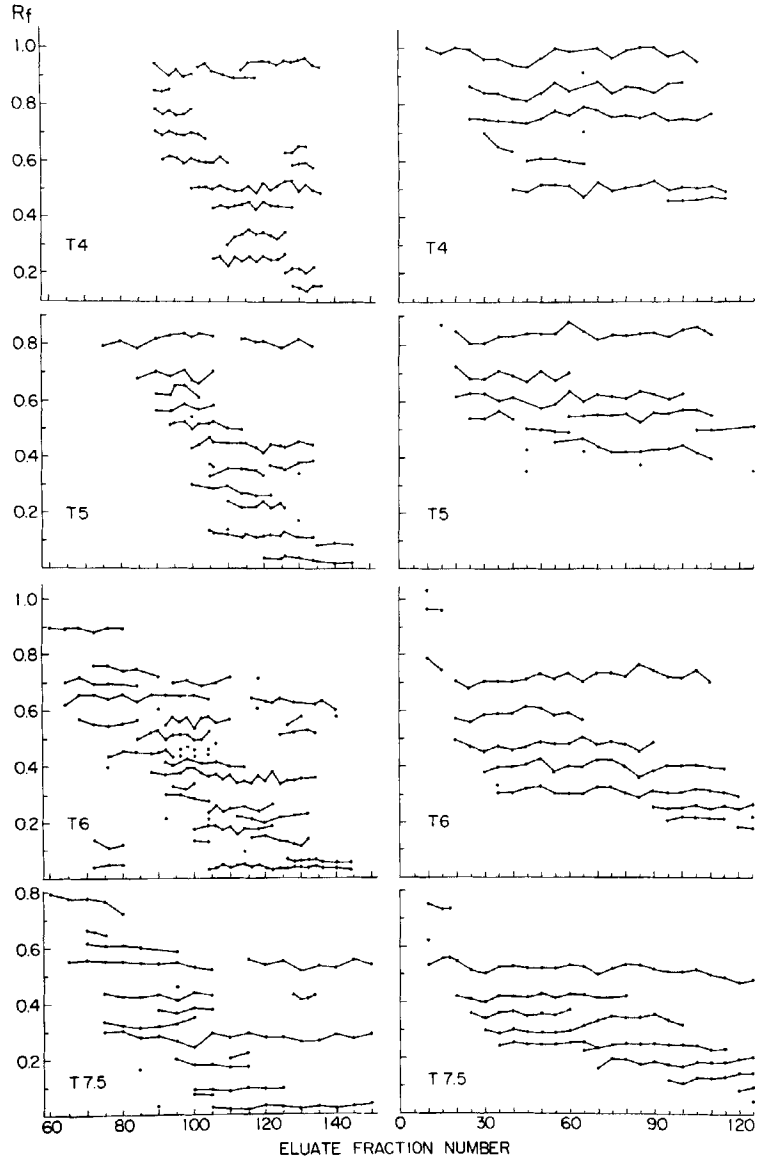

FIG. 9. Two-stage ITPPA-PAGE (left column) and P-G-E–PAGE (right column) fractionation of serum proteins. (Left column) R_f of all bands appearing in PAGE fractionation of the eluate fractions. Load—300 mg/10

of fractionation on the basis of net charge: steady-state-stacking (SSS), including SSS with spacer ions (isotachophoresis, ITP), and isoelectric focusing (IF).

Both methods depend on the minimization of molecular sieving effects, at least in application to proteins. Since the mobilities of proteins are relatively low in general compared to buffer ions, steric hindrance would cause them to be redarded below the value of the lower stacking limit [RM(1,4)] in SSS (*9, 17*). In isoelectric focusing on polyacrylamide gel (IFPA), steric hindrance leads to extension of focusing times and poor resolution in view of the instability of pH gradients with time (*2*). Consequently, the best way to carry out SSS (and ITP) and isoelectric focusing would be in nonrestrictive anticonvective media such as sucrose solutions. However, for analytical purposes, at the microgram scale, PA of minimally restrictive pore size is an easier anticonvective medium to work with. Also, IF in sucrose solution is subject to the problem of precipitation of proteins at their pI values (*2*).

IFPA exhibited the predicted high load capacity (*2*). However, the presence and reactivity of Ampholine, and the instability of pH gradients with time in IFPA (*2*), impose limitations on IF which make it desirable to explore SSS (without spacer ions) as an alternative method.

Steady-state stacking can be applied to analytical (or preparative) fractionation by confinement of either the contaminant (selective unstacking) or of the component of interest (selective stacking) within the stack. However, such situations are rare when the component of interest is embedded in a multicomponent system, which usually comprises molecules with a wide range of constituent mobilities. In the case of multicomponent systems, SSS is a potentially high load fractionation tool at the preparative scale. But it is not readily applicable at the analytical scale where fractionation of the components of a stack would give rise to a continuum of stained regions, i.e., to no detectable resolution.

Theoretically, resolution of the components of a stack can be achieved by introduction of spacers into the stack, i.e., by isotachophoresis (ITP), either at the analytical or at the preparative scale.

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cm² of gel; current—25 mA; elution at 0.8 ml/min. Fractions collected at 10-min intervals. (Right column) P-G-E—PAGE (system B) fractionation of serum proteins. Load—35 mg serum/1.3 cm² of gel; current—6 mA: Fractions were collected at 10-min intervals.

Choice of Buffer System for ITPPA

ITPPA as a fractionation method of high load capacity is dependent on the stacking of the component of interest. The pH at which stacking can be achieved depends on the constituent mobility of the protein compared to the trailing ion (CONSTITUENT 1) (*7*, *9*). Relatively slowly migrating macromolecules can only be stacked at extremes of pH, since only there their mobility exceeds that of the trailing ion. Relatively fast migrating species may still be stacked 1–2 pH units above or below their isoelectric points. If, as is usually the case, the mobility of a protein is unknown, it is recommended to test first at an extreme of pH whether it can be stacked. A convenient test for the stacking of a protein in a particular multiphasic buffer system is to subject it to PAGE in a minimally restrictive (see below) stacking gel (made in phase BETA) (*7*, *9*), together with a suitable tracking dye. After the dye has traversed one-half of the gel length, the migration distance of the dye relative to the gel length is determined. The gel is then fixed and stained; the relative migration distance of the stained protein band is compared with that of the tracking dye. If they are coincident, stacking of the protein has been demonstrated. Alternatively to staining, the protein may be determined by isotope analysis or by activity assay.

The system that exhibits suitably low, lower stacking limits [RM (1,4)] at an extreme of pH can be found in the systems Catalog (*9*) and retrieved from the multiphasic buffer systems output (*9*). If stacking at an extreme of pH has been found, increased selectivity may be obtained by lowering the stacking pH (PH 4) systematically until the above-described type of experiment reveals the retardation of the protein behind the stack. Systematic lowering of the stacking pH (PH 4) can be carried out either between different buffer systems or within a single buffer system by variation of constituent concentrations (page III of the system output for each system, table of STACKING AND UNSTACKING RANGES).

Since high-load capacity within the stack is the prime benefit of ITPPA, it is important to realize that the concentration within the stack increases with increasing concentration of upper gel buffer (phase BETA). An upper limit for the concentration of stacking gel buffer is set by the fact that Joule heating increases and mobility decreases with increasing ionic strength.

Choice of Pore Size in ITPPA

Since stacking of proteins of relatively low mobility is limited by the necessity to exceed the constituent mobility of CONSTITUENT 1 in

PHASE 4 [RM(1, 4)], the protein mobility should not be further decreased by steric hindrance. Therefore, to maintain the high load capacity of a stack, ITPPA of proteins is restricted to a minimally restrictive pore size. Minimal restrictiveness is defined arbitrarily as the same degree of steric hindrance for the protein as offered the tracking dye, determined experimentally by systematic variation of stacking gel concentration, and comparison of relative migration distance between tracking dye and protein as described above. Either a decrease in %T or an increase in %C to 20 or 30% (*21*) or polymerization in synchrony with the gelation of agarose (*20*) can be used to obtain gels of minimal restrictiveness.

Conclusions from Presently Available Evidence on ITPPA at the Analytical Scale

ITPPA at the anaytical scale was introduced recently by Griffith and Catsimpoolas (*12*). Since the properties of the buffer systems used in this study are not known and it was not evident whether the proteins studied were stacked, it was not clear to what degree unstacking and fractionation by molecular sieving (as in conventional PAGE) was superimposed on ITPPA. [Evidence for stacking in these systems has been presented recently (*30*).] The present study shows that Ampholine is effective as a source of spacer ions in ITPPA (Figs. 2 and 3). But Ampholine is apparently not efficient in providing a sufficient variety of ampholytes with constituent mobilities intermediate between those of serum proteins—resolution of serum (Fig. 4) is vastly inferior to that obtained in PAGE. The pI range (Fig. 6) of Ampholine, Ampholine load (Fig. 4), ionic strength of the stacking gel buffer (Fig. 5), and protein load at variable (Fig. 6) or constant (Fig. 7) protein–Ampholine load ratio determine resolution. For each of these parameters there should be an optimum value with respect to resolution of serum proteins. This optimum condition for analytical ITPPA, under the conditions used, appears to be approximately 1 mg protein load, 50 μl, pI 6–8 or 7–10 Ampholine.

In spite of this relative optimum of conditions, the main conclusion from the available data is that Ampholine is not an adequate source of spacer ions for the resolution of a multicomponent system such as serum. Most of the stained patterns are continuous zones as expected from SSS without interposition of spacer ions. It cannot be ascertained from the data to what degree the stained zones are produced by SSS and to what degree by sample overload.

Two-Stage Fractionation of Serum Proteins, Using Preparative-Scale ITPPA followed by PAGE Analysis of the Fractions at Various Pore Sizes

High load capacity in ITPPA was demonstrated at the preparative scale in fractionations with 300 mg serum protein/10.6 cm^2 of gel (or 126 mg urinary Hunter Correction Factor preparation/10.6 cm^2 of gel) up to 200 mg serum protein/1.3 cm^2 of gel. Evidence that these high loads were fractionated effectively derives from the eluate analysis. This shows (Table 3) proteins, characterized by K_R and Y_0, which are not revealed either in one-dimensional analytical PAGE nor in two-stage fractionations consisting of either PAGE or P-G-E at the preparative preparative scale (*16*). Also, as seen from Fig. 9, ITPPA results in the fractionation of proteins characterized by R_f values at various pore sizes, which appear superior to that obtained by P-G-E at less than one-fifth the load. It is not clear, however, whether this appearance of improved resolution is due to achievement of sufficiently high concentrations of components which, with lesser loads (such as in preparative PAGE), would be diluted out beyond detectability, or whether Ampholine binding generates artifactual species. The data shown in Table 1 and Fig. 6 raise the possibility that it might be possible to resolve complex multicomponent systems by two-stage fractionations, where the preparative scale tolerates very high loads so that minor components of the system can be detected during the second stage (carried out on equal amounts of each component). This approach has the further advantage that the two fractionation stages vary in principle of operation, the first being largely responsive to molecular net charge, the second to size. However, there remain at present grave problems of component identification at the second fractionation stage (*16*).

APPENDIX I Procedure for Polymerization of Acrylamide in Synchrony with the Gelation of Agarose

An agarose–PA gel was prepared by a modification (*25*) of the procedure of Peacock and Dingman (*20*). Using the polymerization conditions of PAGE (*19*), which give rise to polymerization within 10 min, the temperature of an agarose–acrylamide–Bis mixture was lowered concomitantly with the vinyl polymerization to achieve the gelation of agarose within the same period of time.

A 1.1% agarose solution was refluxed for 10 min, using an air con-

denser and magnetic stirring. The flask was then placed into a water bath maintained at 45 to 50°C. An acrylamide polymerization mixture (10 ml 4% acrylamide, 0.4% Bis solution, and 5 ml 4× concentrated stacking gel buffer (system B)) was prepared and deaerated (*19*). It was then brought to 45°C with stirring and combined with 4.5 ml of the agarose solution, 0.5 ml of catalyst solution (0.6% ammonium persulfate, 0.01% RN) and 40 μl of TEMED. The mixture was immediately dispensed into tubes of a Polyanalyst (Buchler Instruments Div.) apparatus maintained at room temperature and containing lower buffer. Coolant flow at 0°C and illumination were then initiated simultaneously.

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