3. ADVANCED ANALYTICAL TECHNIQUES

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New analytical concepts are being considered for use in clinical and biomedical research laboratories. Some of these concepts, after initial testing proves their feasibility, will be developed into prototype systems which can be evaluated under realistic laboratory conditions. Ultimately it is hoped that successful developments will be produced by commercial firms to aid in the process of technology transfer.

3.1 High-Resolution Liquid Chromatographic Systems

Various disease states may give early warning of their onset or their continuing presence by subtle changes in excretion levels of molecular constituents in physiologic body fluids. Prompt medical treatment based on these early symptoms may prevent the disease from developing, retard the progress of the disease, or at the very least, minimize its debilitating effects. Detection and monitoring of these early symptoms will require that research and clinical laboratories be equipped with automated high-resolution analytical systems that will identify and quantitate many specific molecular constituents. To attempt to fill the need for specificity and high sensitivity, liquid chromatographic (LC) systems are being developed. These high-resolution systems, having the advantages of unattended operation and minimal sample preparation, enable a large number of important nonvolatile metabolites to be monitored with minimal use of skilled technical labor using automated data processing methods.

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3.1.1 Glycoprotein carbohydrate analyzer

We have continued routine use of gradient elution chromatography for the analysis of the protein-bound carbohydrates mannose, galactose, and fucose. Further studies have been performed on a LC system using a shorter column and employing isocratic elution to attempt to analyze the protein-bound carbohydrates in about 4 hr.

Shortened analysis time for protein-bound neutral carbohydrates. Preliminary information published previously[1,2] suggested the possibility of analyzing serum protein-bound mannose, galactose, and fucose by isocratic elution using a 0.22- by 50-cm column. While separation had been demonstrated using reference standard sugars in about the same relative amounts as expected in serum samples,[1,2] analysis of a serum sample had not been attempted. Illustrated in Fig. 3.1 is the separation of a hydrolyzed serum sample using isocratic elution (0.168 M boric acid, 0.0075 M ammonium acetate, pH 7.7). Note the large peak at the beginning of the chromatogram representing breakthrough of nonadsorbed materials. The massive size of this peak relative to the three carbohydrate peaks results in considerable overlap of this peak with those of mannose and fucose, making quantitation of both carbohydrates difficult because of baseline uncertainties. Ethanol, used in precipitation of serum protein, was found to contribute to the size of the first peak, and resulted in a decrease in the size of the first peak when it was removed completely. However, baseline slope was still excessive.

In addition to excessive slope of the baseline, there are at least two peaks whose elution positions overlap that of fucose (see Fig. 3.1). This causes additional uncertainties in measurement of the small peak area

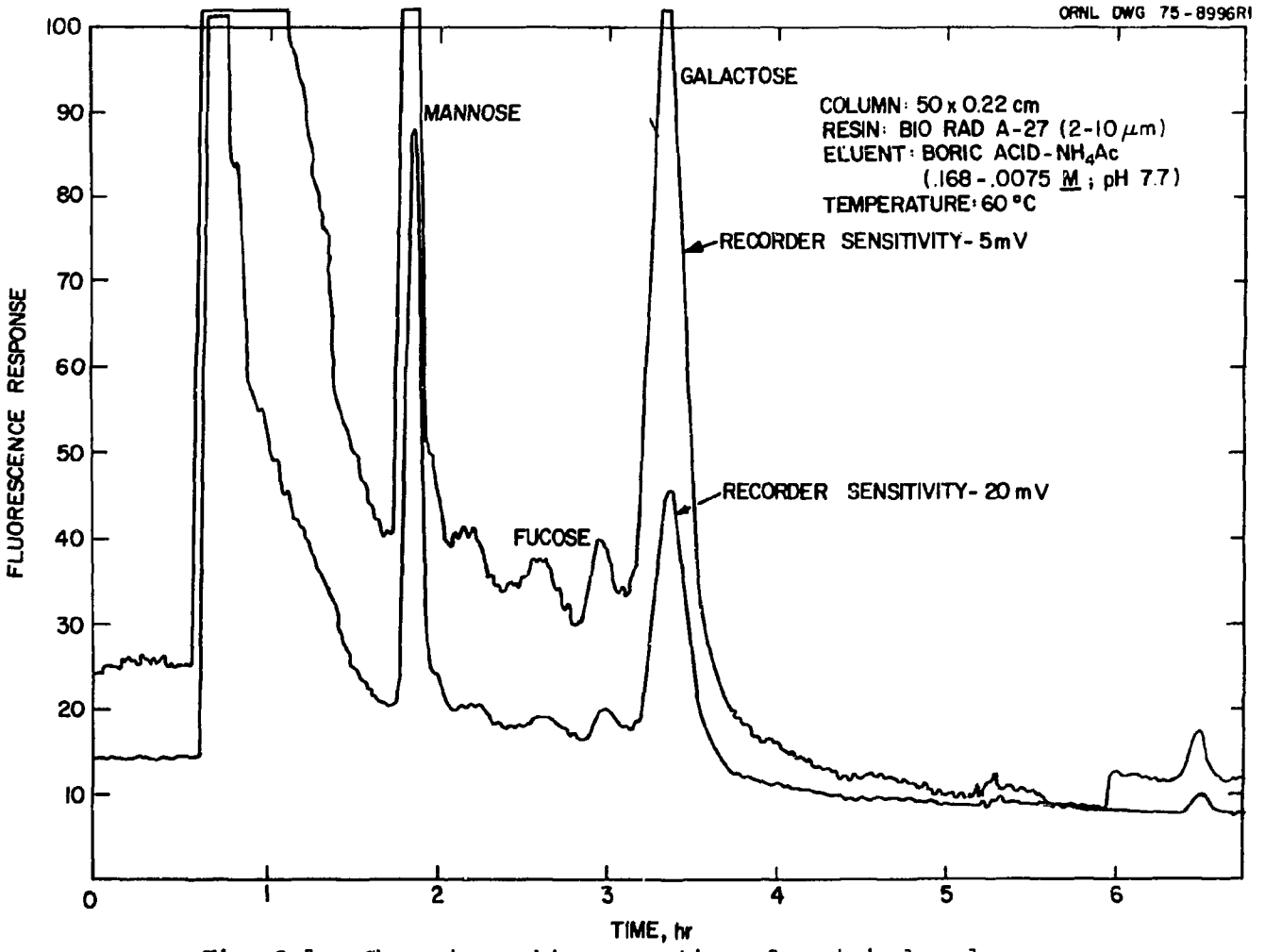

Fig. 3.1. Chromatographic separation of protein-bound mannose, fucose, and galactose in a hydrolyzed serum sample using isocratic elution. Note the baseline slope due to the massive peak at the front of the chromatogram.

for fucose. The origin and identity of these interfering peaks have not yet been determined; however, reversion of mannose, galactose, or fucose under these elution conditions has not been eliminated as a possibility, although not observed for standard carbohydrates. Accurate quantification of the relatively small fucose peak is not feasible with the peak overlap and the baseline slope shown in Fig. 3.1.

Measurement of sialic acid. The methyl pentose fucose is one of the terminal sugars of glycoproteins. The second terminal compound found in carbohydrate side chains of glycoproteins is n-acetylneuraminic or sialic acid. The former compound, with its terminal methyl group, is hydrophobic in nature, whereas the latter, with its terminal polar carboxyl group, is hydrophilic.

Evaluation of the efficacy of measurements for both fucose and sialic acid as biochemical markers of cancer is desirable. However, hydrolysis and chromatographic conditions for these two compounds differ substantially. Sialic acid is destroyed under hydrolysis conditions required to liberate fucose (1 N HCl for 4 hr), whereas fucose is not released under hydrolysis conditions which liberate sialic acid nondestructively (0.1 N H_2SO_4 at 80°C for 1 hr).[3,4] This makes it virtually impossible to analyze both compounds in the same sample.

Elution studies of reference n-acetylneuraminic acid revealed that it could be analyzed chromatographically using ammonium acetate--acetic acid buffer at pH 4.4 with detection by the cerate oxidimetric detector system.[2] Linearity of both peak area and peak height was observed for amounts of sialic acid ranging from 6.5 to 39.1 μg eluted from the anion exchange column (see Fig. 3.2).

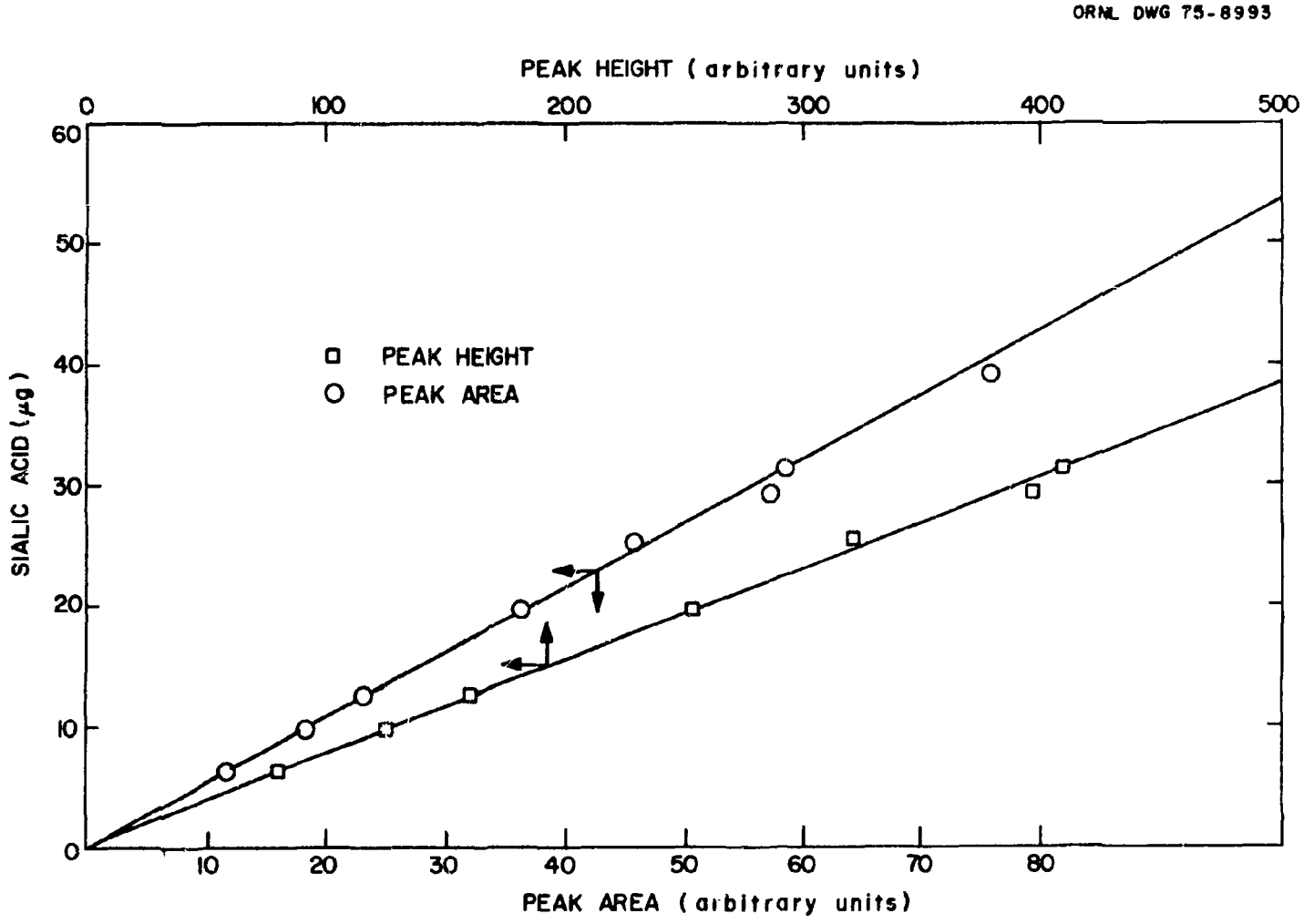

Fig. 3.2. Linear response of the cerate oxidimetric system is demonstrated for sialic acid eluted from a chromatographic system.

Stability of sialic acid to hydrolysis conditions. Five samples of n-acetylneuraminic acid were exposed to hydrolysis conditions of 0.1 N sulfuric acid at 80°C for 1 hr. The samples were cooled to room temperature and adjusted to approximately pH 4 with 1 N sodium hydroxide. The samples were analyzed by LC with an average recovery of 97.2% of the sialic acid and a coefficient of variation of 2.2%, indicating these hydrolysis conditions apparently would not affect sialic acid recovery.

Analysis of protein-bound sialic acid in a serum sample. Protein precipitated from a 0.5-ml sample of serum was subjected to the hydrolysis conditions described above. The hydrolyzed sample, after being adjusted to approximately pH 4, was placed on an anion exchange column (0.22 by 100 cm) and eluted with a concentration gradient of acetate buffer. One major chromatographic peak was eluted at about 7 hr; its elution position corresponded with the elution position observed for reference n-acetylneuraminic acid. Four other relatively minor peaks were observed along with the strong peak usually observed at the front of the chromatogram due to sample breakthrough.

Few extraneous peaks were observed in the chromatogram with gradient elution, and thus conditions were sought which would enable faster analysis of sialic acid without the necessity of column regeneration. A number of different concentrations of the acetate buffer were tested to find conditions which would enable separation of the sialic acid peak from the large peak occurring at sample breakthrough. Illustrated in Fig. 3.3 is the chromatogram obtained for a hydrolyzed normal serum sample eluted with 0.2 M ammonium acetate--acetic acid buffer at 60°C. Measurement of the area of this peak and comparison with standards as shown in Fig. 3.2 yielded a sialic acid content, in this serum, of 423 μg/ml. Further studies will be

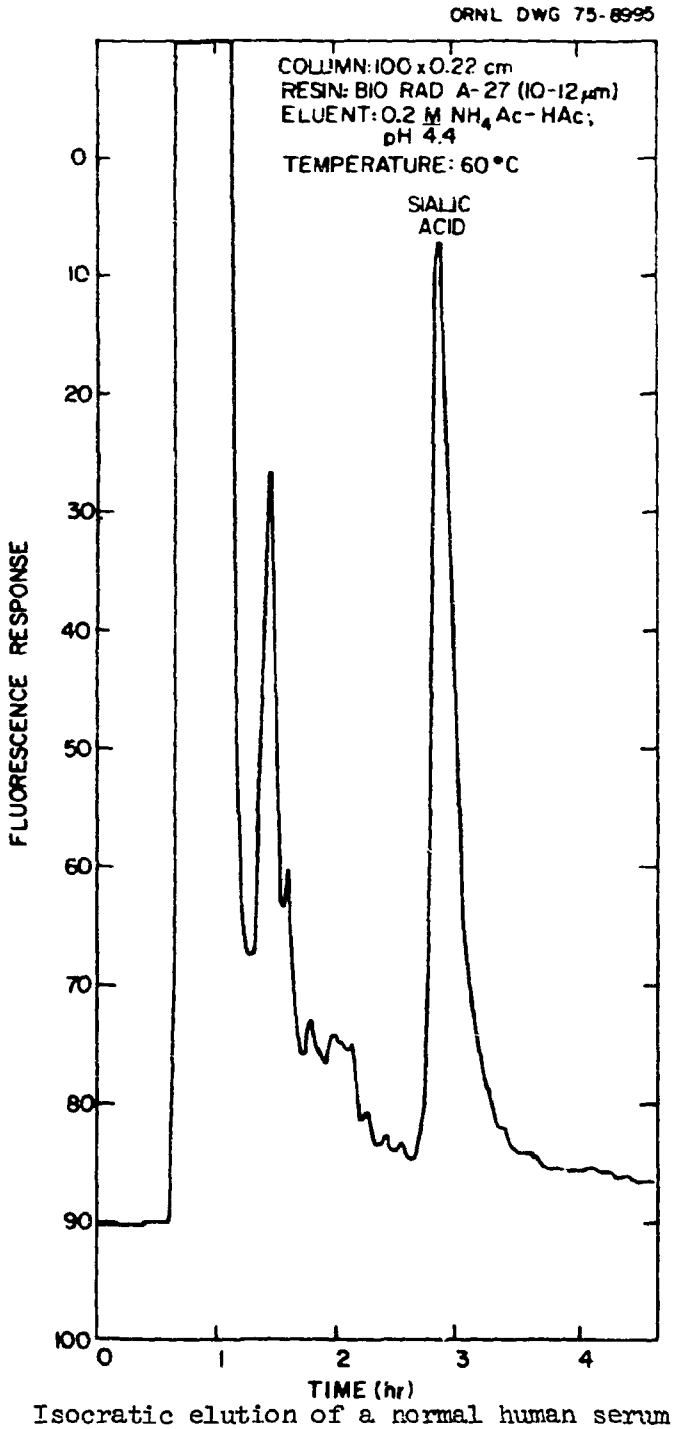

Fig. 3.3. Isocratic elution of a normal human serum sample which was hydrolyzed under mild conditions designed to release only sialic acid. Gradient elution, performed previously, had demonstrated that reference n-acetylneuroaminic acid coeluted with the peak designated as sialic acid.

performed with isolated glycoproteins to determine if recovery of sialic acid is linear with the amount of protein.

3.1.2 Experimental results and applications

We have continued analytical separations studies on physiologic fluids to demonstrate useful applications of high-resolution liquid chromatographic systems and detectors that have been developed. Emphasis is continuing on the separation and analysis of protein-bound carbohydrates in sera from breast cancer patients.

Analysis of neutral protein-bound carbohydrates. We have continued the routine analysis of protein-bound mannose, galactose, and fucose in sera from breast cancer patients as described previously.[1,2] Multiple samples have been analyzed for patients in different stages of the malignancy. Clinical data describing the physical status of the disease and including serum protein analysis were available for some of the patients (see Table 3.1 and Fig. 3.4); additional data of this nature will be available at a later date.

The solid rectangle shown in Fig. 3.4 represents the range of values (fucose/protein, total hexose/protein) found for normal female subjects by LC analysis of hydrolyzed sera.[1,2] The data shown in this figure for six patients whose clinical status was available are interesting. Three of the patients had their malignancy described as either in full or partial remission at the first sampling and were all within the range of normal values. Two of the three patients were described as having either disease progression or relapse at their next sampling (see patients 1 and 6, Table 3.1) and their analytical data had become abnormal (see Fig. 3.4). The third patient (No. 5, Table 3.1) was described as initially in partial

Table 3.1. Serum protein-bound neutral carbohydrates in patients with breast cancer

Patient number	Sample identification	Mannose (mg/100 ml)	Fucose (mg/100 ml)	Galactose (mg/100 ml)	Protein (mg/ml)	Clinical disease status
1	1523	46.8	5.3	51.6	80	Surgery - complete remission
	2065	57.2	7.5	58.3	75	Relapse
	2498	36.5	4.4	34.8		
2	352	47.2	5.6	43.0		
	1471	42.8	3.4	39.8	79	Improving
	1604	50.2	3.8	46.1	78	Partial remission
	2403	48.4	5.2	41.4		No change
3	1261	42.4	6.7	46.5	83	No change
	1669	49.9	5.9	50.0	75	Partial remission
4	1595	52.3	5.8	59.4	99	Complete remission
	2177	51.8	6.3	47.3	78	Progression
5	686	49.6	6.7	51.2	82	Partial remission
	1366	59.9	7.5	57.5	52	No change
	1854	50.4	8.9	57.3	75	Relapse
	1860	52.1	7.8	50.0		
6	740	46.7	5.4	45.2	86	No change
	1373	43.6	5.9	40.9	95	Partial remission
	1935	48.4	6.0	48.4	61	Relapse
	2466	35.9	8.6	35.8		
	3431	40.0	6.5	38.5		Progression
7	1312	65.9	6.6	62.1	79	No change
	1463	46.9	5.5	51.2	75	No change
	1599	39.3	5.2	37.1	81	No change
	2253	40.4	3.9	36.9		
8	1602	54.2	7.8	59.2		
	1815	46.7	6.6	51.6		
	3183	32.8	5.3	33.1		
9	680	44.6	6.9	46.8		
	1033	45.7	7.4	49.8		
	1229	52.5	10.0	60.0		
	3144	39.5	5.6	43.0		
10	1704	44.2	8.7	44.0		
	3315	43.1	5.8	55.3		

Table 3.1 (continued)

Patient number	Sample identification	Mannose (mg/100 ml)	Fucose (mg/100 ml)	Galactose (mg/100 ml)	Protein (mg/ml)	Clinical disease status
11	699	43.9	8.5	52.8		
	913	44.1	8.4	52.6		
	1295	44.5	8.0	42.7		
	1878	38.8	7.3	34.8		
12	2402	42.4	4.5	40.9		
	2742	30.6	6.1	35.6		
13	391	50.6	5.7	49.5		
	646	42.2	5.9	44.2		
	1015	51.9	6.1	57.9		
	1464	41.7	4.9	42.2		
14	346	51.6	5.9	54.0		
	1064	48.6	6.8	42.3		
	1622	44.7	5.7	42.7		
	2929	44.1	5.9	46.2		
15	268	46.0	7.6	60.9		
	1323	46.6	7.1	47.9		
16	342	71.8	8.2	74.4		
	1397	31.6	4.0	31.5		
17	362	42.5	4.4	50.0		
	887	39.1	4.4	42.3		
	1360	38.3	4.6	38.2		
	1903	46.2	4.0	45.5		
	3543	39.6	4.9	40.0		
18	454	53.9	6.7	62.1		
	885	47.3	8.4	66.8		
19	787	49.6	6.3	51.8		
	1272	40.0	4.6	40.9		
20	1758	55.1	4.5	59.1		
	2138	40.1	4.8	35.7		
21	2364	50.2	7.2	52.8		
	2932	47.6	6.8	48.5		
22	1232	49.4	6.4	56.1		
	1509	53.2	7.5	56.2		
23	392	57.4	5.3	59.3		
	671	51.4	4.9	55.0		

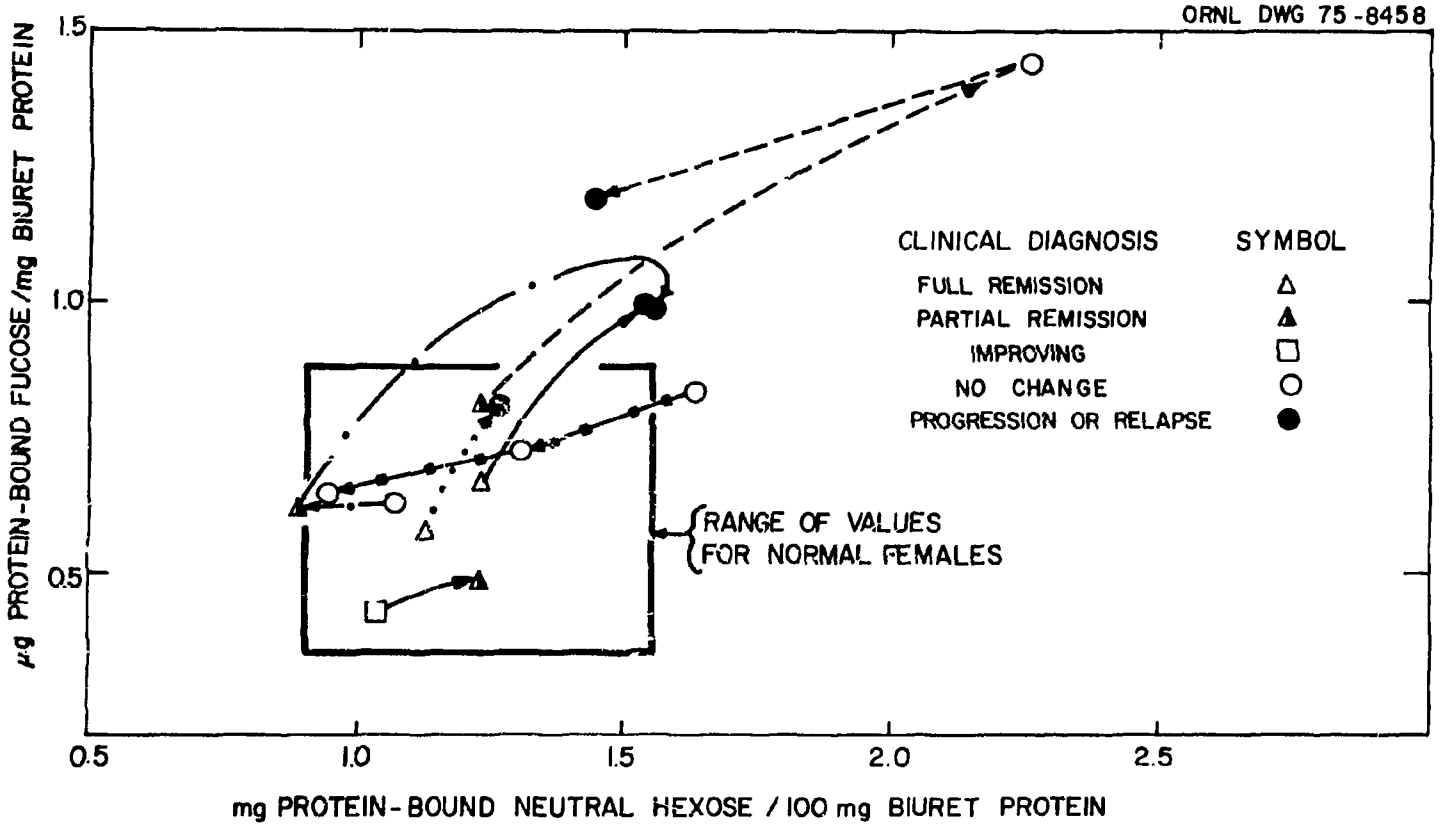

Fig. 3.4. The effect of physical status of breast cancer patients on serum protein-bound fucose and mannose plus galactose.

remission, followed by "no change" and then suffered a relapse at the third sampling. Our analysis showed a change had occurred between the first and second samplings, and this change was magnified by a large drop in serum protein. The analytical change had preceded a clinically observable difference in the disease which is necessary for a biochemical marker of malignancy to be useful. However, patient 4 went from full remission to disease progression, while her fucose-to-protein and hexose-to-protein ratios remained within normal bounds. The remaining three patients (2, 3, and 7, Table 3.1) displayed little change in their clinical status and their analyses also remained normal; patient 7 did appear to be improving as indicated by a decline in both fucose-to-protein and hexose-to-protein ratios, although clinically there was no apparent change in her status.

3.2 Blood Sample Preparation System

N. E. Lee and J. E. Mrochek

Genetic mutation is commonly monitored by the determination of variant cellular isoenzymes using electrophoretic separation techniques coupled with staining of specific proteins. Meaningful results necessitate the analysis of large numbers of blood samples which, in turn, require a large amount of technical manpower to prepare cellular hemolysates for analysis. The blood sample preparation system described here is designed to separate whole blood into plasma and cells, to wash the cells, collecting samples of each fraction, and to facilitate the preparation of a lysed cell sample for electrophoretic analysis.[5,6] Evaluation of early prototypes by personnel at ORNL and the University of Michigan demonstrated the feasibility of adapting all steps in the procedure to a sample preparation system;

however, problem areas were uncovered which required additional development effort.

3.2.1 Experimental system

A major problem uncovered by electrophoretic studies of samples prepared by the early prototypes has been the inability to fully sediment the stroma or cell debris in the lysate at the maximum rotor speed (2300 rpm) available in these early systems. Personnel at the University of Michigan found that centrifuging the system-prepared hemolysates at 18,000 rpm for 20 min did result in suitable samples for electrophoretic analysis, whereas samples without the high-speed spin yielded undesirable smearing of the electrophoretic patterns. Interlaboratory visits and communication between ORNL and University of Michigan personnel suggested that more efficient lysing procedures and more vigorous mixing of the hemolysate and the organic phase (carbon tetrachloride) might enhance the sedimentation of cell debris.

A third-generation prototype system (Figs. 3.5 and 3.6) has been fabricated to provide for more vigorous mixing of the cells during the washing and lysing steps of the procedure. The new prototype employs a rotating seal to enable a partial vacuum to be maintained above the liquids in the sample compartments of the rotor, causing air bubbles to be drawn into the liquids through the exit channels. In addition, a solenoid valve can be activated to cut off the vacuum and introduce bursts of air through the same exit channels to also enhance mixing.

Another problem area uncovered during work with the early prototypes was an undesirable liquid transfer during acceleration. The rotor was redesigned[7] (Fig. 3.6) with modified syphon channels to eliminate this

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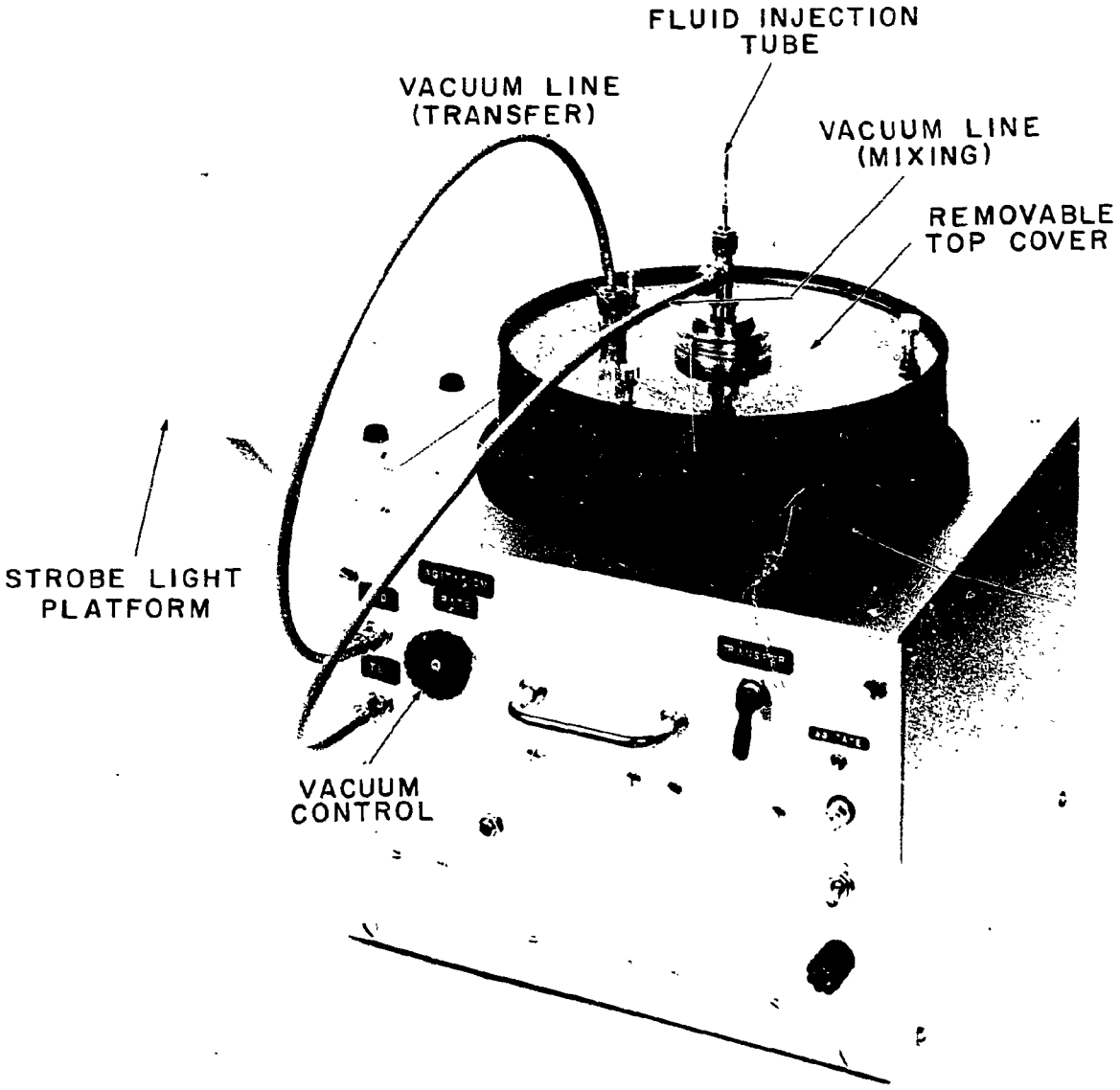

Fig. 3.5. Front view of improved blood sample preparation system.

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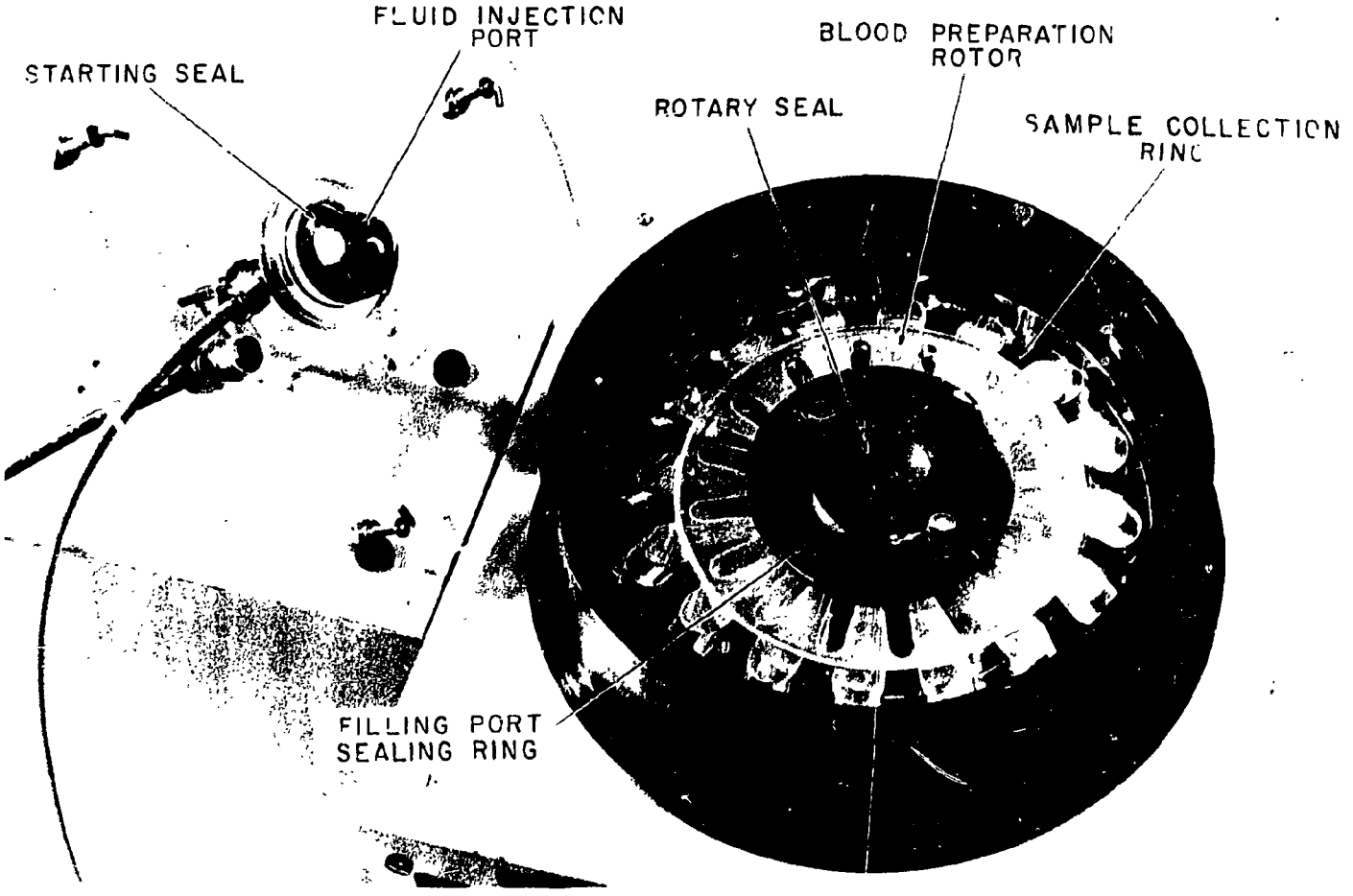

Fig. 3.6. Close-up view of new rotor designed for use in the blood sample preparation system.

problem. However, due to increased efficiency within the rotor, coupled with decreased cross-sectional area of the syphon channels, additional problems have surfaced. The rotor is fabricated of Plexiglas and, due to the inherent nature of its use, must be thoroughly sealed. The seal must withstand vigorous cleaning as well as contact with carbon tetrachloride, which is required during the lysing process to aid in sedimenting the stroma to yield hemolysates that will provide clean electrophoretic band patterns. As a result of this repeated contact of the rotor chambers with small quantities of carbon tetrachloride during lysing, severe crazing has occurred in the extreme outer regions of the chambers and along the cross-sections of the syphoning channels. To overcome these problems, alternative materials (i.e., polycarbonate) are being considered for rotor fabrication.

Another severe limitation encountered with this prototype system has been the elimination of the in situ rotor cleaning step. Operator workload has been increased excessively; however, a separate rotor washing station may overcome this problem.

3.2.2 Studies of system variables

Several lysing procedures were evaluated to optimize hemolysate preparations. The standard procedure used with this system has been to lyse the red blood cells by dynamically introducing 16 cc of distilled water into the rotor containing the cells; accelerating, agitating, and braking several times to facilitate mixing, followed by the dynamic addition of 8 cc of 0.05 M HCl made up in 0.005 M KH_2PO_4; repeating the mixing procedure; and adding 4 cc of carbon tetrachloride and again repeating the mixing procedure, accelerating to maximum rpm and centrifuging for 15 to 20 min to remove stroma. The following alternative lysing procedures were tested:

(1) Doubling the volume of carbon tetrachloride to 8 cc.

(2) Increasing the volume of water to 20 cc and carbon tetrachloride to 8 cc, while eliminating HCl/KH_2PO_4.

(3) Increasing the volume of water to 24 cc and HCl/KH_2PO_4 to 8 cc, while eliminating carbon tetrachloride.

(4) Adding 8 cc of 0.05 M KH_2PO_4 (pH 4.0) together with 16 cc of water and 4 cc of carbon tetrachloride.

(5) Adding 24 cc of 0.005 M KH_2PO_4 (pH 6.0) and 4 cc of carbon tetrachloride.

The results obtained by each of these procedures were equally poor. A commercial hemolysing agent, Saponin, was obtained, and concentrations of 0.01%, 0.1%, 0.25%, 0.5%, 0.75%, and 1.0% by weight in distilled water were prepared. The standard lysing procedure described above was followed except that Saponin-water replaced water. Samples of the hemolysates from each run were then subjected to starch gel electrophoretic analysis which showed promise for concentrations of Saponin equal to or greater than 0.5%.

3.2.3 Electrophoretic results on experimentally prepared hemolysates

The criterion for judging the quality of the hemolysates obtained from this system is electrophoresis and subsequent staining of various isoenzyme systems. And although activity for several isoenzyme systems has been demonstrated, totally acceptable hemolysates have not yet been prepared using prototype systems. This is primarily due to insufficient aggregation of the cell stroma when carbon tetrachloride is added. When the standard lysing procedure, described above, is used with either 0.5%, 0.75%, or 1.0% Saponin solution, and the resulting hemolysate is subjected to an additional

20 min in a Sorvall centrifuge at 18,000 rpm, the heretofore nonsedimented stroma form a nicely packed bead and electrophoretic results are excellent.

The enzymatic activity of lactate dehydrogenase (LDH) as obtained directly from the system and after additional Sorvall centrifugation is evidenced by a comparison of starch gel electrophoresis results. Figure 3.7 (top) clearly indicates a high degree of band smearing for samples from the system, while (bottom) a clean, well-defined band resulted after Sorvall high-speed spin. A further comparison in Fig. 3.8 (top, system; bottom, Sorvall) clearly shows the advantage of the Sorvall centrifugation step in eliminating band smearing when starch gel electrophoresis for malic dehydrogenase (MDH) is performed.

For the initial studies conducted with this prototype system, only about 30% of the total capability for air injection was utilized and samples were centrifuged at 2200 rpm for 20 min after lysing. Later studies have utilized the full capability for air injection, and centrifugation has been extended to 30 min at 2600 rpm. Acrylamide gel electrophoresis and subsequent staining of the LDH isoenzymes revealed improved resolution and decreased band smearing without additional Sorvall high-speed spinning.

3.3 Automated Elution Electrophoresis

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Earlier reports[8-10] of the development of automated elution electrophoresis demonstrated the feasibility of attaining the following advantages over the electrophoresis systems most commonly used in clinical laboratories (i.e., electrophoresis on cellulose acetate strips):

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ORNL DWG 75-8731

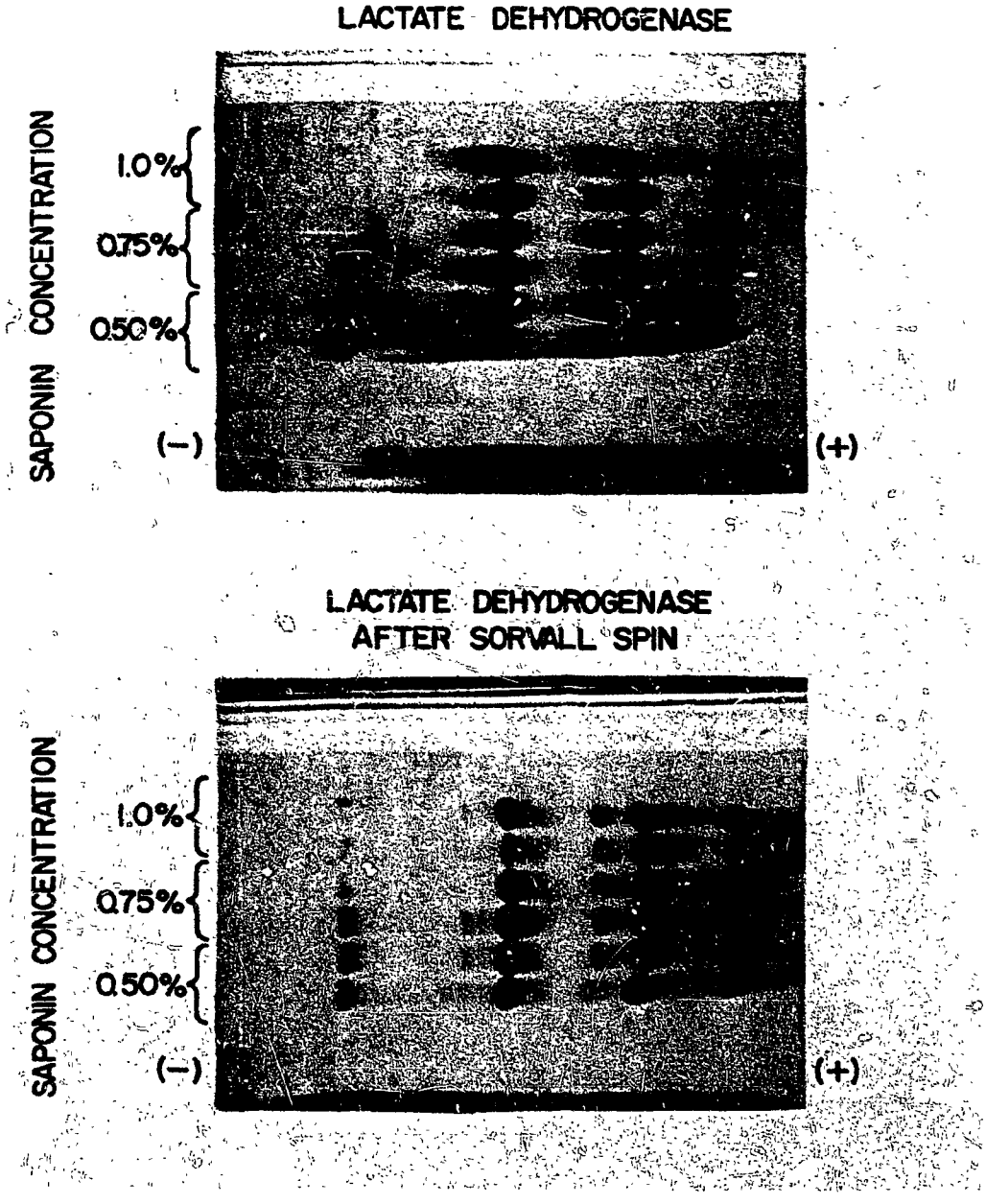

Fig. 3.7. Lactate dehydrogenase isoenzyme determination by starch gel electrophoresis of cell hemolysates prepared using the blood sample preparation system. Note the improvement in electrophoretic pattern after sedimenting stroma by high-speed centrifugation.

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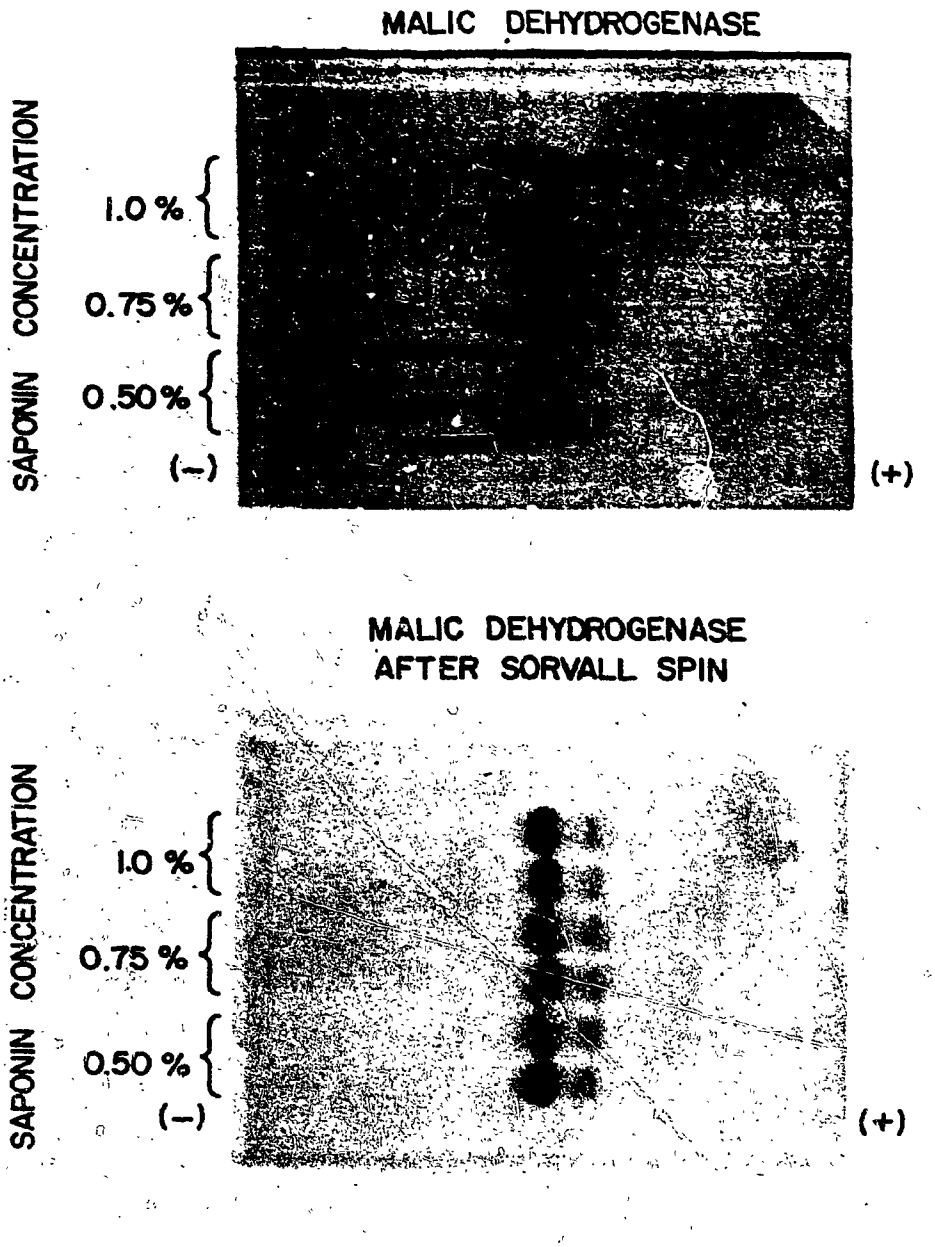

Fig. 3.8. Malic dehydrogenase isoenzyme determination by starch gel electrophoresis of cell hemolysates prepared using the blood sample preparation system. Note the improvement in electrophoretic pattern after sedimenting stroma by high-speed centrifugation.

(1) A simplification of the operational sequence required for each analysis[10] to a rapid and easy sample introduction;

(2) Usage of high voltage gradients (with forced cooling of the column) and forced elution, resulting in a reduction of the time required for analysis to approximately 1 hr (typically 4 to 24 hr);

(3) An increase in resolving capability; and

(4) Automation of the results with quantitation of the separated constituents in the eluate by a flow photometer or colorimeter.

While the prototype system successfully demonstrated the operating concept, much work remained to develop an elution electrophoretograph appropriate for use in routine clinical analyses. Also, no attempt was made to optimize the buffer system or other operating parameters.

Recent development efforts have been directed toward defining routine operating procedures which would give reproducible results with acceptable resolution. Some investigation of different column designs, packing materials, and buffer systems has been performed. All work has been done with serum protein samples.

3.3.1 Experimental system parameters

Columns. Both horizontal and vertical column arrangements were investigated. The horizontal system is shown in Fig. 3.9; the same column was used in the vertical system with minor adaptations to allow electrolysis gases to escape. Column diameters of 3.4 mm and 4.2 mm were used, and effective lengths of bed packing varied from 20 to 40 cm (protein migration was always anodic for the buffer systems used, and sample introduction septa were accessible at both the center and cathodic end of the packing).

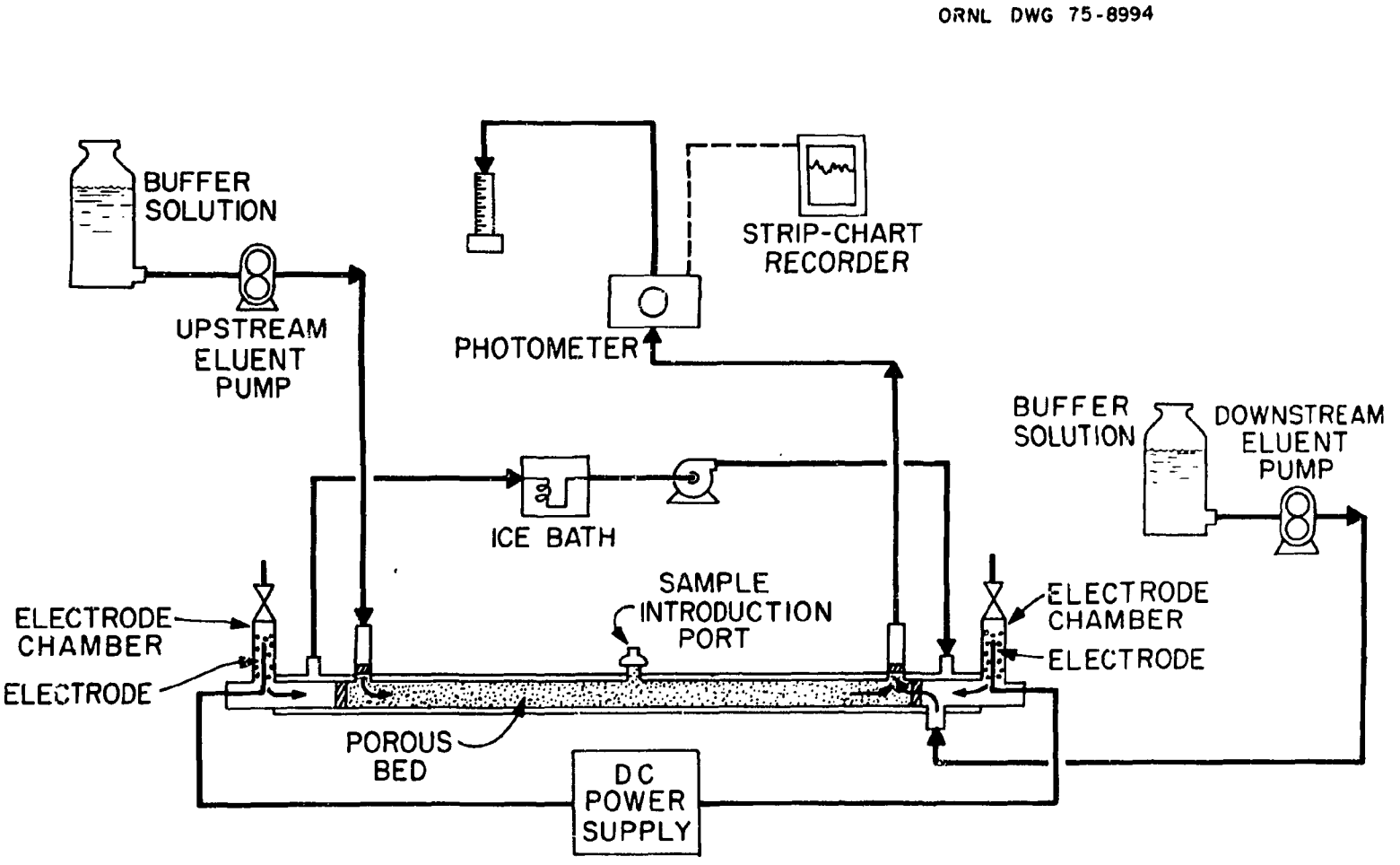

Fig. 3.9. Schematic diagram of the elution electrophoresis system.

Packing materials. Two packing materials were tried, Bio-Gel P-2 and Ultrogel AcA 54. The addition of 4% agarose to the usual polyacrylamide gel matrix distinguishes the Ultrogel from the Bio-Gel. The exclusion range of the Bio-Gel was 100 to 1800 daltons, while the range for Ultrogel was 6,000 to 70,000 daltons based on measurements with globular proteins. The molecular weight of the smallest serum protein, albumin, is approximately 69,000 daltons. Therefore, porosity of the packing material was of negligible importance.

Buffer systems. Two buffers with varying molarities and ionic strengths were tried. A Tris-barbital--sodium-barbital buffer which has produced high resolution with other electrophoresis systems was tested at pH 8.6 for ionic strengths of 0.01, 0.1, and 0.3. A phosphate buffer was used routinely at pH 8.6 and at 0.125 and 0.25 M concentrations.

The barbital system (see Table 3.2) was unsatisfactory for two reasons. First, the system was stable only in the pH range of 8.2 to 8.9 and formed precipitates outside that range. Thus, the pH changes occurring in the electrode chambers degraded the buffer, and the resulting precipitates were washed through the eluate monitoring system. The second disadvantage of the buffer system was its high uv absorbance which also varied significantly with pH changes in the electrode chambers. Both effects resulted in artifact peaks demonstrable by electrophoresis in the absence of a protein sample.

The sodium phosphate buffer gave a much lower background absorbance with approximately one-half the changes in absorbance observed with the barbital buffer, even though larger pH changes occurred in the electrode chambers. This buffer was acceptable for routine use.

Table 3.2. Variation in buffer absorbance and pH values during elution electrophoresis

	pH	uv absorbance	Percent transmittance
Tris-Barbital--Sodium-Barbital			
Ionic strength, 0.01			
Before electrophoresis	8.6	0.290	51.0
After electrophoresis - upstream	9.2	0.340	45.5
- downstream	8.3	0.340	45.5
Ionic strength, 0.3			
Before electrophoresis	8.6	0.94	11.5
Sodium Phosphate			
0.25 M			
Before electrophoresis	8.5	0.04	91.5
0.125 M			
Before electrophoresis	8.5	0.02	95.5
After electrophoresis - upstream	10.4	0.05	89.5
- downstream	7.5	0.04	91.0

Voltage gradients and elution rates. Electrophoresis was performed with applied voltages ranging from 200 to 1500 V (producing from 5 to 40 mA of current) for time periods of 15 to 90 min. Elution flow rates varied from 0 to 15 ml/hr across the column during electrophoresis. Elution flow rates after hydrostatic electrophoresis were routinely 6 ml/hr. Protective flows away from the anodic electrode chamber were routinely 2 ml/hr.

3.3.2 Visualization studies

A 0.1% solution of bromophenol blue dye was used in a ratio of 1:1 with both 4.3% albumin preparations and whole serum samples. Introduction of the albumin-dye sample into the column clearly demonstrated the need to extract the microsyringe from the septum very slowly. Otherwise, sample rather than packing would fill the void created by forcing the syringe into the column. Samples drawn into the introductory port washed very slowly back into the column during a run and produced broad, trailing peaks.

Initially, sample introduction and elution were performed without electrophoresis. Separation of stained albumin from excess dye was easily achieved in this manner. Three color regions were observed with whole serum samples, probably marking excess dye, albumin, and globulins. It was observed that sample bandwidths depended, as noted, on sample introduction and that the protective flows directed out of the electrode chambers were effective. Samples either introduced or eluted from column areas outside the packing were observed to mix excessively.

In some experiments, elution flows were used to resolve dyed albumin from excess dye, followed by the establishment of hydrostatic conditions in

the column. Electrophoresis at 1000 V using the phosphate buffer produced no visual movement of dyed albumin within 45 min.

3.3.3 Status of development

Reproducible results have been obtained by these authors with this system, but not with resolution that will compete with electrophoresis on cellulose acetate strips as originally demonstrated.[8-10] No significant differences in resolution were obtained by varying the parameters described within the ranges defined. It is probable that performance-governing parameters for this system have not yet been identified. Presently, efforts are being made to define conditions favorable for electrophoretic mobility. These efforts include studies of the resistances for gradients associated with the dc-field applied across the column as well as the current-carrying properties of different buffer systems. Studies with a Tris-borate buffer are planned since borate may make glycoproteins in serum more negatively charged by complexing carbohydrates in these macromolecules. This higher negativity might then make them more susceptible to electrophoretic migration.

3.4 Centrifugal Chromatography

A new, removable-column rotor has been developed which greatly enhances the experimental flexibility and operation of the prototype system for centrifugal elution chromatography.[9,11] New inorganic immunochemical sorbents have been prepared which appear to overcome problems (pluggage, etc.) exhibited by gels when used with the centrifugal elution chromatograph system. The new immunosorbents have been used with the removable-column rotor to study affinity separations of IgG from human serum.

Additional studies will emphasize the determination of several serum proteins (e.g., human immunoglobulins) simultaneously from a single sample.

3.4.1 Development of a removable-column rotor

The new, removable-column rotor (Fig. 3.10) accepts up to eight chromatographic columns. The assembly consists of a plastic center insert for partitioning the sample and eluent stream among the separate flow channels. A flow channel consists of a chromatographic column followed by a flow-through cuvet. The final element is a retainer clip with center screw which is used to compress and secure the assembly within the rotor. Center inserts presently available permit operation in either the four-column or eight-column mode. Four-column sets are available with 0.238-, 0.318-, 0.397-, or 0.476-cm ID by 10 cm in length. The assembled rotor, containing four chromatographic columns, is shown as a part of the centrifugal elution chromatograph in Fig. 3.11.

3.4.2 Preparation and testing of inorganic immunochemical sorbents

In a previous report,[1] affinity separations were discussed which utilized a gel (Sephadex G-25) as the support for an immobilized antibody (antihuman IgG). Although affinity separations of human IgG from human serum were achieved, the gel exhibited unsatisfactory characteristics with regard to flow of eluent through the gel bed. Under the centrifugal forces used to produce the desired eluent flow rate, the bed tended to compress and bridge, forming both densely packed and void regions within the flow channel. After searching the literature, it was concluded that inorganic supports such as alumina or titania might be more suitable for use with our centrifugal elution system. Such particles would be more rigid than gels and less expensive than controlled-pore glass.

ORNL PHOTO 2161-75

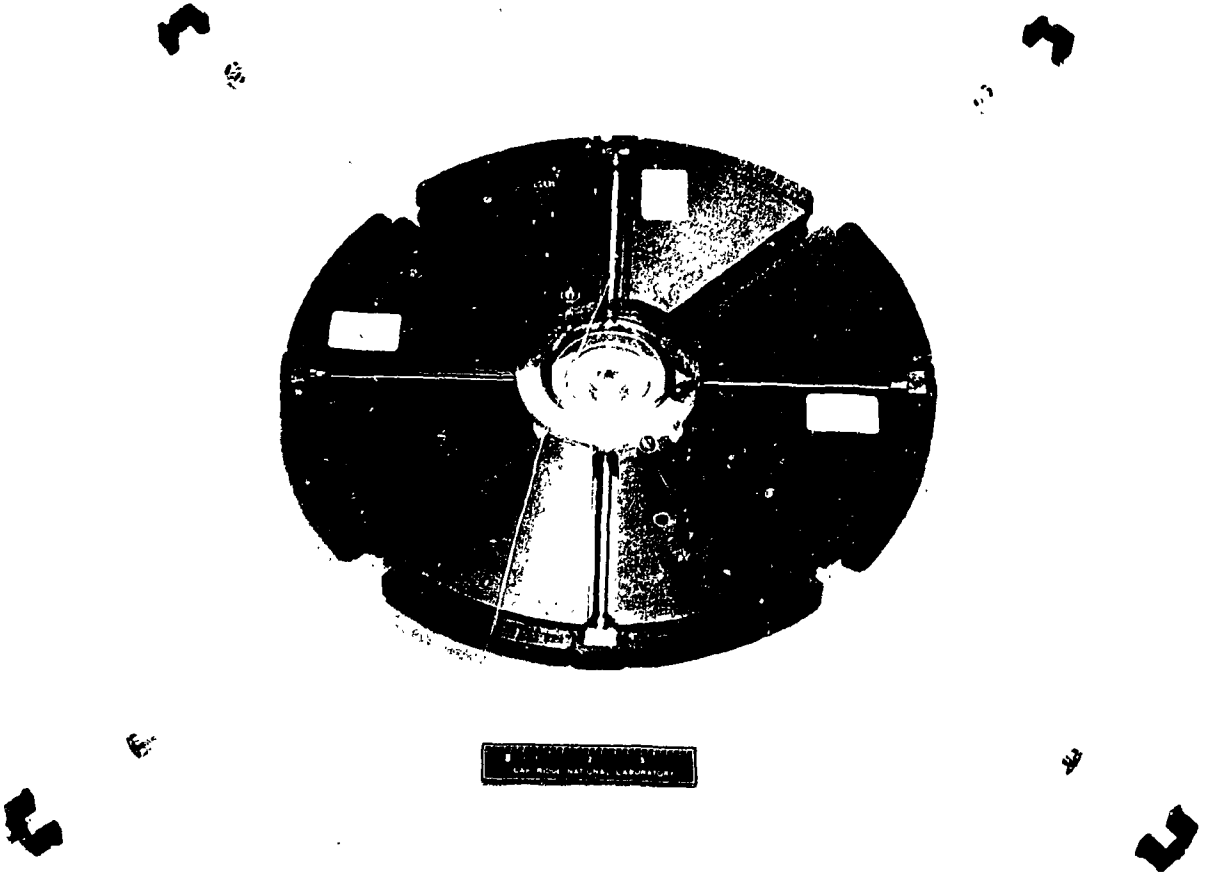

Fig. 3.10. Removable-column rotor for centrifugal elution chromatography. Components include center insert, columns, flow-through cuvets, and retainer clips.

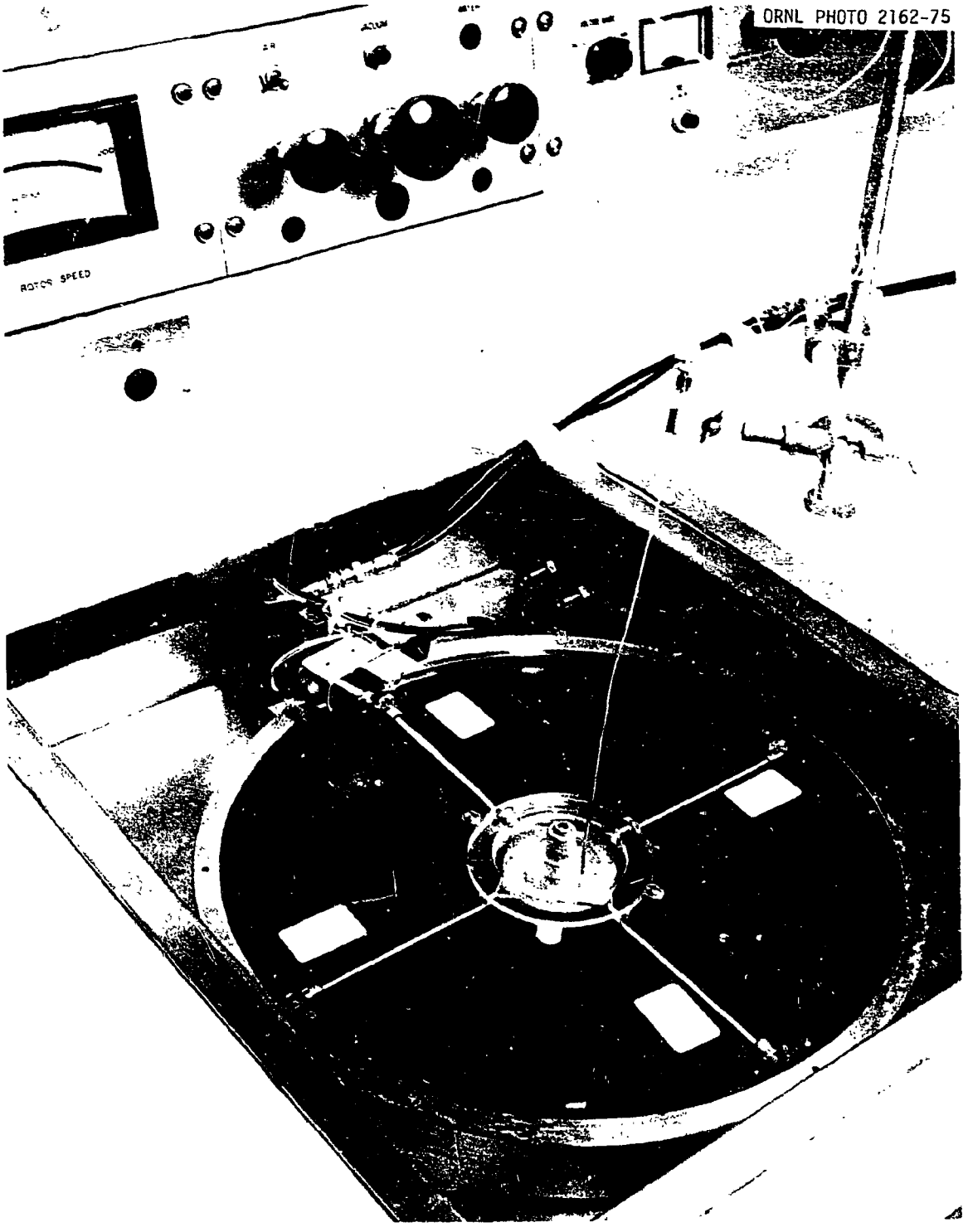

Fig. 3.11. Assembled rotor containing four columns, shown as a part of the centrifugal elution chromatograph.

The first immobilization technique attempted was adsorption of antihuman albumin on 70- to 100-mesh alumina. Although we were able to adsorb approximately 22 O.D. units of antihuman albumin per gram of alumina, the immobilized antibody exhibited insufficient immunological activity. A technique was then developed for covalent attachment of proteins to alumina using cyanogen bromide activation of the support. Alumina (70 to 100 mesh) was washed first with distilled water to remove brown coloration and then with 0.1 M sodium phosphate buffer, pH 7.0. Fifteen grams (dry basis) of alumina and 20 ml of 0.1 M sodium phosphate buffer were placed in a beaker in an ice bath. The solution pH was maintained at 11.0 during addition of CNBr (4.5 g). The reaction mixture was then moved to a coldroom and gently rotated for 3 hr. The alumina was washed with 0.1 M sodium phosphate buffer to remove adsorbed but noncovalently bound protein. Finally, the immunosorbent was reequilibrated with the eluting buffer for the ensuing experiments in centrifugal elution chromatography.

3.4.3 Experimental results on the affinity separation of IgG from human serum

The immunosorbent prepared by covalent attachment of antihuman IgG to alumina was slurry packed into four columns 0.318 cm ID by 10 cm long. The rotor was assembled as previously described and placed in the centrifugal elution chromatograph housing. The rotor was spun at 400 rpm with a steady flow of 0.1 M sodium phosphate buffer (pH 7.0) through the four columns. A 40-μl volume of human serum, diluted to 1 ml with buffer, was dynamically injected into the splitting vanes of the rotor insert. The nonadsorbed proteins are eluted as a large peak on the chromatogram (Fig. 3.12). A second peak consisting of nonspecifically adsorbed proteins was eluted with

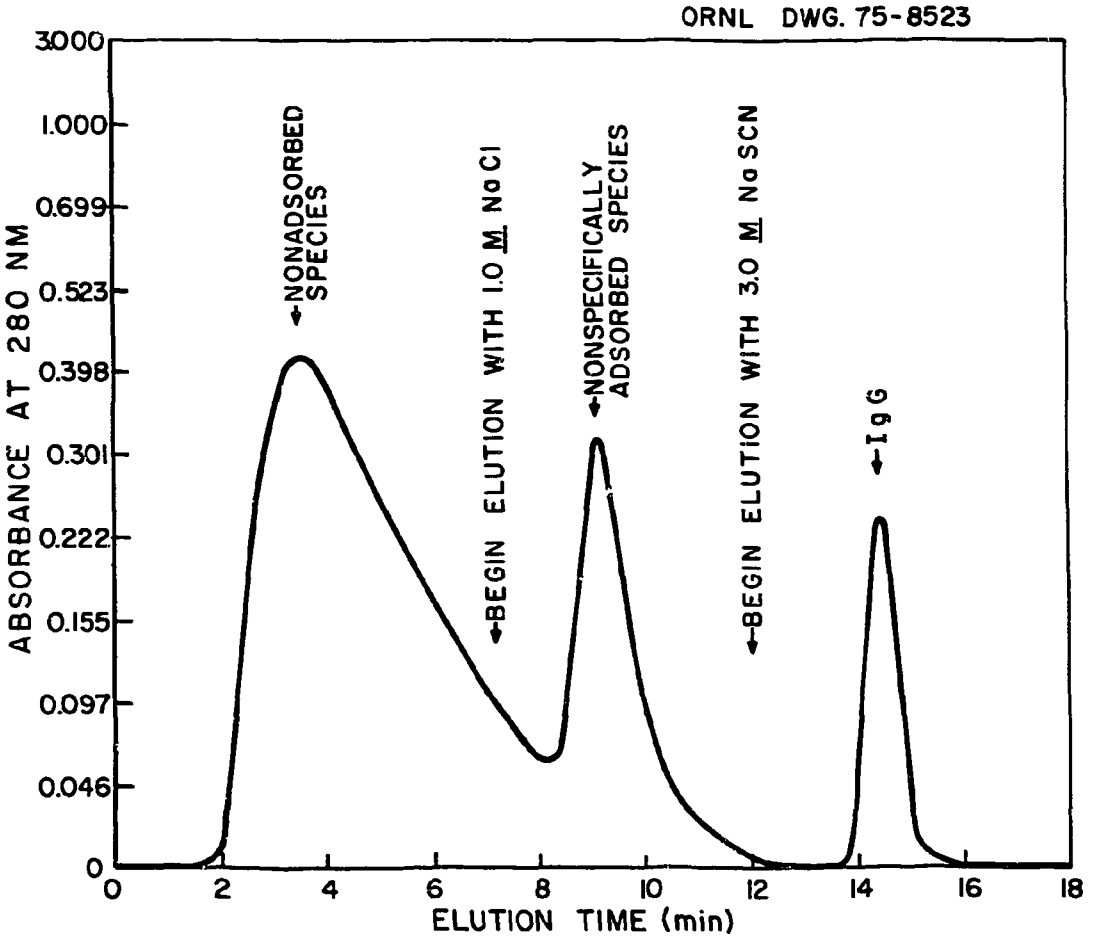

Fig. 3.12. Affinity chromatogram for the separation of human IgG from normal human serum on a centrifugal elution chromatograph. Immunosorbent was antihuman IgG covalently attached to alumina.

1.0 M NaCl in phosphate buffer (pH 7.0). Finally, the peak for the immunosorbed protein of interest, IgG, was eluted with 3.0 M NaSCN. Serum sample sizes studied were 100, 50, 25, and 10 μl. The 10-μl sample produced a sizeable IgG peak (third peak, Fig. 3.12), reflecting excellent sensitivity of the detection system.

Additional experiments will be performed to optimize the sample size of human serum. The capability of the system for performing rapid determinations of several different serum proteins (e.g., human immunoglobulins) from a single sample will be demonstrated.

3.5 References for Section 3

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