ISOTACHOPHORESIS OF

HUMAN CEREBROSPINAL FLUID

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CONTENTS

SUMMARY

Isotachophoresis, an electrophoretic technique which separates and concentrates charged molecules in a discontinuous electrolyte system according to mobility, was used to examine human cerebrospinal fluid (CSF) proteins from different acute and/or chronic neurological diseases.

The analytical separation of CSF proteins was performed in the 0,45 mm diameter capillary of the LKB 2127 Tachophor. Separated zones were detected by ultra-violet absorption.

Qualitative as well as quantitative work was performed, with the latter being used to show linear relationships between albumin concentration and peak width, peak height and peak area. A linear relationship was also found between immunoglobulin concentration and peak area. The area measurements were used in examining CSF protein separation profiles where the integrity of the blood-CSF (blood-brain) barrier could be determined as well as intrathecal immunoglobulin synthesis.

From the qualitative studies, CSF protein patterns of acute viral meningitis, acute bacterial meningitis, cryptococcal meningitis, presumptive multiple sclerosis, Guillain-Barré syndrome and subacute sclerosing panencephalitis (SSPE) differed from the normal CSF profile. These variations were usually confined to the immuno-

globulin region. However, in acute bacterial meningitis and cryptococcal meningitis, there was evidence of blood-brain barrier damage with the resultant leakage of serum proteins into the CSF.

The different CSF isotachophoretic profiles were useful in the diagnosis of some of the diseases - e.g. SSPE.

The CSF protein profile could be divided into two main UV-absorbing regions - the front running peak (FRP) region consisting of substances migrating well ahead of the second protein region. The position of some of the proteins found in the CSF - e.g. albumin, transferrin, α_2 macroglobulin, haptoglobin, IgG, IgM and IgA, were determined. Two of the FRP were identified as folic acid and uric acid. The significance of an increase in CSF uric acid levels was examined.

The MES/Ammediol leading electrolyte was used in this study. However, it was found that the FRP and proteins were regulated by another leading ion, the chloride ion present in the CSF sample as sodium chloride. The chloride ion was found to take over the function of the leading ion with the chosen leading ion, MES, acting as a spacer between the FRP and the main protein region.

CHAPTER I

PRINCIPLES AND THEORY OF ISOTACHOPHORESIS

1.1 INTRODUCTION

Isotachophoresis may be described as an electrophoretic technique used in the separation and concentration of charged molecules of the same sign according to their mobilities in a closed discontinuous system where the equilibrium (steady state) once formed, is a dynamic one.

A brief historical review of isotachophoresis is presented in this Chapter, together with the principles of the technique. These include the separation and concentration of zones and the sharpening of zone boundaries.

The influence of salts from the sample on the functions of the leading electrolyte are described.

The parameters that affect the dynamic equilibrium, once this has been established, are given.

The theory of the Kohlrausch regulating function is described briefly.

1.2 HISTORICAL REVIEW

A German chemist, F.W. Kohlrausch (1897) showed that in isotachophoresis the concentration of ions at the boundary between two salt solutions is related to their effective mobilities. This relationship is described by the Kohlrausch regulating function (See Appendix A). Kendall and White (1924) described the separation of metal ions by what they termed the ionic migration technique. However, it was only in the 1960's that the principle of isotachophoresis was seriously applied to the separation of ionic solutions, by Konstantinov (in Haglund, 1970; Hjalmarsson and Baldesten, 1981), Vestermark (1967), Ornstein (1964) and Davis (1964). The latter two authors made use of the concentrating effect of this technique in their discontinuous electrophoresis experiments. The sample was separated and concentrated in a narrow zone between two buffers (isotachophoresis) and then further separated by zone electrophoresis.

As the zones separated by isotachophoresis are in immediate contact with each other, Vestermark (in Haglund, 1970; Hjalmarsson and Baldesten, 1981) suggested the use of non-UV absorbing substances of intermediate mobility to act as "spacers" between the zones.

Since the development and production of the commercial isotachophoretic equipment (Tachophor) by LKB-Produkter AB, this technique has been used in the analysis of a wide variety of

substances including organic and inorganic ions, nucleotides, amino acids, peptides, proteins and fatty acids. Isotachophoresis has been used in many disciplines : pollution and quality control, pharmaceutical and food industry, medicine and research.

During its development, this technique has been known by several names : ionic migration technique (Kendall and White, 1924), ion-mobility analysis (in Haglund, 1970), cons electrophoresis (in Haglund, 1970), displacement electrophoresis (Martin and Everaerts, 1967, 1970), moving boundary method (in Haglund, 1970), steady state stacking (Ornstein, 1964) and ionophoresis (in Hjalmarsson and Baldesten, 1981). In 1970 a number of workers in this field, including Haglund (1970), agreed to adopt the name isotachophoresis (Greek : Iso - equal; tacho - speed).

1.3 PRINCIPLES

Isotachophoresis will occur when an electric current is applied to a sample of unknown constitution, sandwiched between electrolytes, one of which consists of high mobility ions and the other of low mobility ions. Once the sample ions have been separated, a steady state is established which is dependent on a number of parameters.

1.3.1 Electrolytes

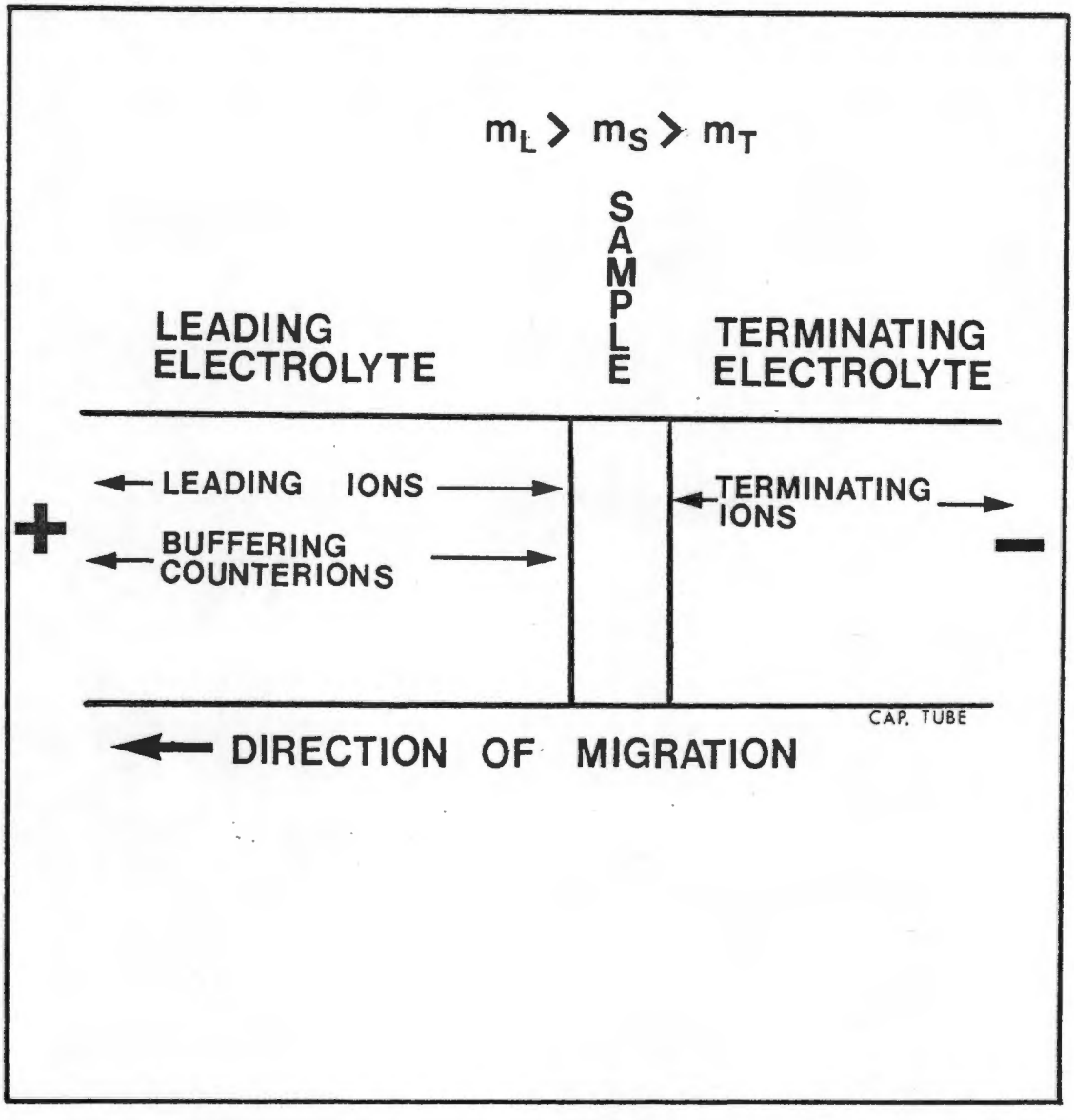
Analytical isotachophoresis takes place when an electric field is applied to a tube containing two different electrolytes. The leading electrolyte contains the leading ion of high mobility and is negatively charged in an anionic system and positively charged in a cationic system. The terminating electrolyte contains the terminating ion of mobility less than the leading ion and sample ions to be separated and it is also negatively charged. The ionic concentration of both these electrolytes is approximately 10^{-2} - 10^{-3} molar. The buffering ion, or counterion, is added to the leading electrolyte and it is chosen so that its buffering capacity is in the pH range of the two electrolytes used. It has an opposite charge to the terminating and leading ions and thus, in an anionic system, is positively charged. The sample ions are introduced between the leading and terminating electrolytes and have intermediate mobilities. They are negatively charged in an anionic system and the concentration is variable. In the case of proteins, the concentration is usually lower than the leading ion. Figure 1.1 illustrates the position of the electrolytes and sample in relation to each other.

1.3.2 Separation Mechanism

The mechanism of initial separation of the ions occurs in the following manner. When a constant current is applied to the capillary containing only the leading and terminating

FIGURE 1.1

The schematic arrangement of the leading, terminating, buffering counterions and sample in the capillary (Cap. tube) at the start of an isotachophoretic experiment where the mobility of the leading ion (m_L) is greater than the sample (m_S) which in turn has a mobility greater than the terminating ion (m_T).


$m_L > m_S > m_T$
SAMPLE
LEADING ELECTROLYTE
TERMINATING ELECTROLYTE
LEADING IONS
BUFFERING COUNTERIONS
TERMINATING IONS
+
−
CAP. TUBE
DIRECTION OF MIGRATION

electrolytes, the leading ions of greater mobility than the terminating ions move away from the leading and terminating ion boundary and a zone depleted of ions is created.

Thus, a high potential gradient is formed in this zone and forces the terminating ions to follow directly after the leading ion and migrate at the same velocity. Due to the low mobility of the ions of the terminating electrolyte, this solution must decrease in concentration near the leading electrolyte, in order to establish a high electric field strength (Baldesten, 1980). Figure 1.2 illustrates this point.

When a sample is introduced between the two electrolytes, the sample ions move initially at different speeds, by the moving boundary separation process (Mikkers and Everaerts, 1981), until they are separated in order of their net mobilities, with the faster-moving sample ions in contact with the leading electrolyte. At this stage a steady state (dynamic equilibrium) is reached. The separated sample zones now migrate with equal velocities, in immediate contact with each other. The sample zones are either diluted or concentrated in accordance with the Kohlrausch regulating function (Figure 1.3).

Some sample ions may have mobilities greater than the leading ion and so move ahead of the leading ion boundary in an

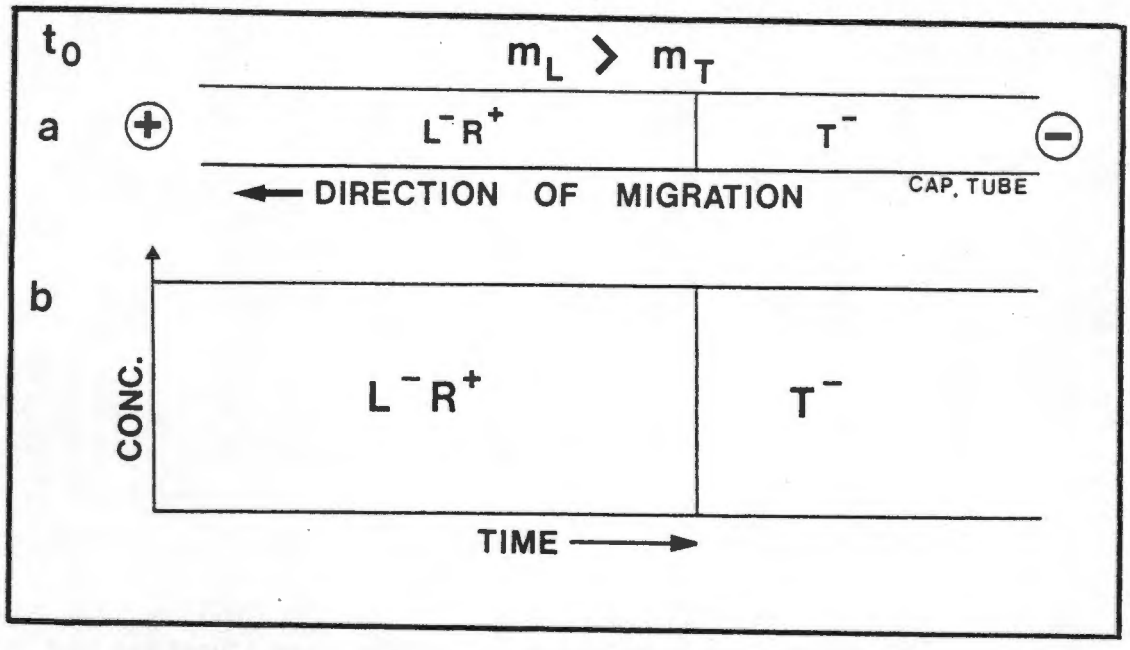
FIGURE 1.2

t_o (a) Initial conditions in the capillary tube (Cap. tube) with a boundary between the electrolyte solutions L^-R^+ and T^-, where L^- is the leading ion with a mobility (m) greater than the terminating ion (T^-). R^+ is the buffering counterion.

(b) The concentration of L^- and T^- at the start.

$t_{eq.}$ (a) Conditions in the capillary tube (Cap. tube) at equilibrium. The boundary of L^- and T^- has moved from its initial position.

(b) The concentration of L^- and T^- at equilibrium where the T^- concentration has decreased in accordance with the Kohlrausch regulating function.


t_0
$m_L > m_T$
a
L^-R^+
T^-
DIRECTION OF MIGRATION
CAP. TUBE
b
CONC.
L^-R^+
T^-
TIME

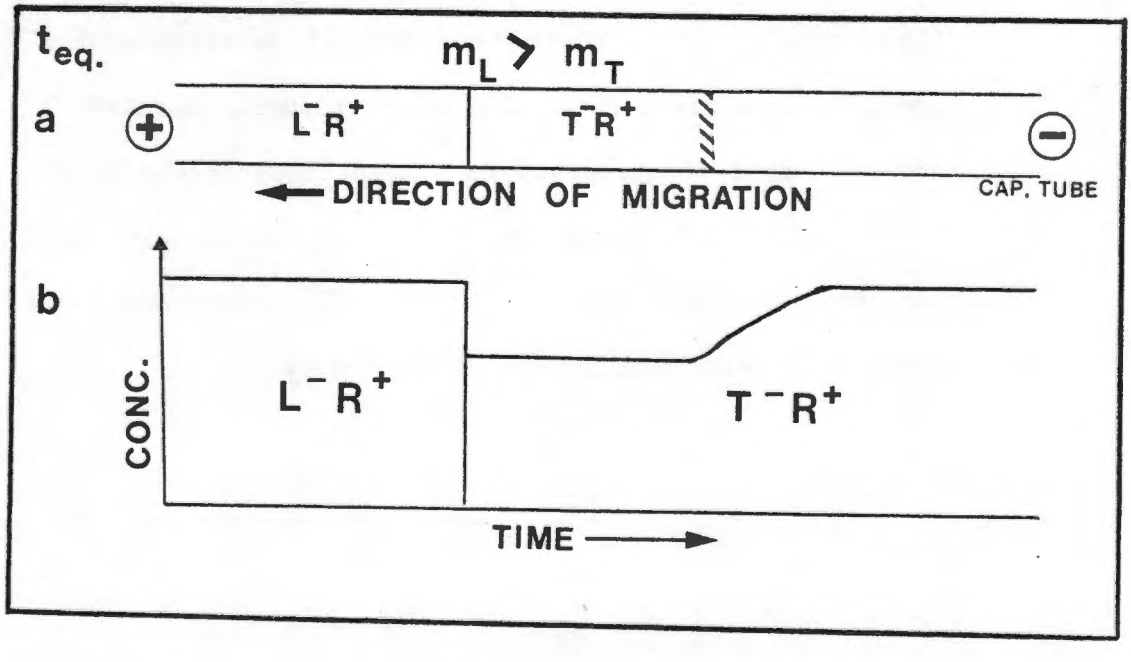
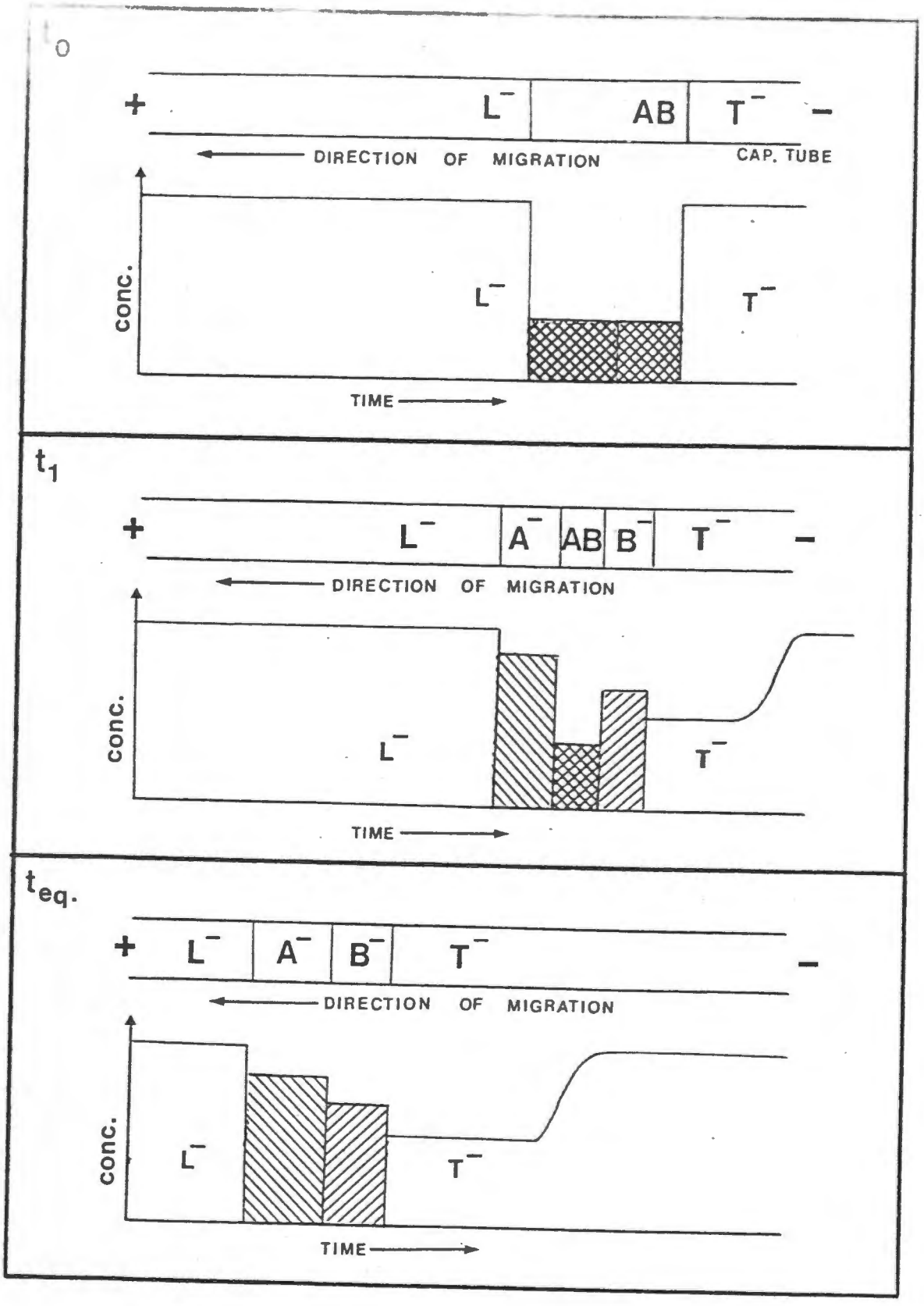

$t_{eq.}$
$m_L > m_T$
a
L^-R^+
T^-R^+
DIRECTION OF MIGRATION
CAP. TUBE
b
CONC.
L^-R^+
T^-R^+
TIME

FIGURE 1.3

The process of separation of sample constituents A and B which have mobilities intermediate to those of L^- and T^-. Consider A^- to have a mobility greater than B^-.

t_o is the position and concentration of the L^-, T^- and AB when the sample is introduced into the capillary tube (Cap. tube). At t_1 there is a partial separation of A^- and B^- with a mixed zone, AB, still present. Both the A^- and B^- sample constituents have been concentrated at the L^-/A^- and B^-/T^- boundaries. At t_{eq}. a steady state has been reached and the A^- and B^- constituents are completely separated and are concentrated.


t0
+
L⁻
AB
T⁻
−
DIRECTION OF MIGRATION
CAP. TUBE
conc.
L⁻
T⁻
TIME
t1
+
L⁻
A⁻
AB
B⁻
T⁻
−
DIRECTION OF MIGRATION
conc.
L⁻
T⁻
TIME
teq.
+
L⁻
A⁻
B⁻
T⁻
−
DIRECTION OF MIGRATION
conc.
L⁻
T⁻
TIME

"out of stack" configuration (Mikkers and Everaerts, 1981). These sample ions are then separated zone electrophoretically in the leading electrolyte. Sample ions of mobility less than the terminating ion also move in an "out of stack" configuration by zone electrophoresis behind the terminating ion boundary in the terminating electrolyte. These are called trailing ions.

1.3.3 Parameters

Once a steady state has been reached, a number of parameters, some of which may be used in detection, can be defined.

1.3.3.1 pH Value

There is a general pH increase in the anionic system towards the terminating electrolyte (Everaerts and Routs, 1971). This is due to the negative ion of the most acidic component usually having the highest net mobility as well (Haglund, 1970). The pH difference occurring between consecutive zones may range from one tenth of a pH unit to several pH units (Everaerts and Routs, 1971), and is regulated by the buffering counterion (Verstermark, 1970). (According to Martin and Everaerts (1970), each separated zone will determine its own pH and have no influence on the pH of other zones.)

The hydroxonium (H_3O^+) and hydroxyl (OH^-) ions may play

an adverse role in the ionization of electrolytes if their ratio is not balanced. These ions also influence the conductivity, but their effect is only serious at a high or low pH (Everaerts and Routs, 1971).

1.3.3.2 Concentration

There is a decrease in the concentration of the separated zones towards the terminating electrolyte. This is in accordance with the Kohlrausch regulating function. It is the concentration of the leading electrolyte that determines the concentration of all zones behind it.

1.3.3.3 Temperature

The temperature within the system increases towards the terminating electrolyte. This is a stepwise increase, occurring at the zone boundaries, due to the potential gradient within each zone increasing in order to maintain the ions at a constant velocity throughout the system.

Joule heat is a product of current (I) and resistance (R) and, as the current remains constant but the resistance increases with each decrease in zone concentration, the heat produced must increase (Joule heat = I^2R).

1.3.3.4 Mobility

The separated zones are arranged in order of decreasing

mobility as described by the Kohlrausch regulating function.

1.3.3.5 Field Strength

The field strength is increased towards the terminating electrolyte as the concentrations of the zones behind the leading electrolyte are reduced. According to the equation $V^* = m.E^*$, where the velocity (V^*) in the isotachophoretic system is constant and the mobility (m) decreases, it is the field strength (E^*) that must increase to maintain the equilibrium (Hjalmarsson and Baldesten, 1981).

1.3.3.6 Conductance and Resistance

Each zone has a defined electrical resistance (R) and, therefore, a defined conductivity (C) ($C = 1/R$). The resistance increases towards the terminating electrolyte due to a decrease in ionic concentration and, thus, the conductivity is decreased.

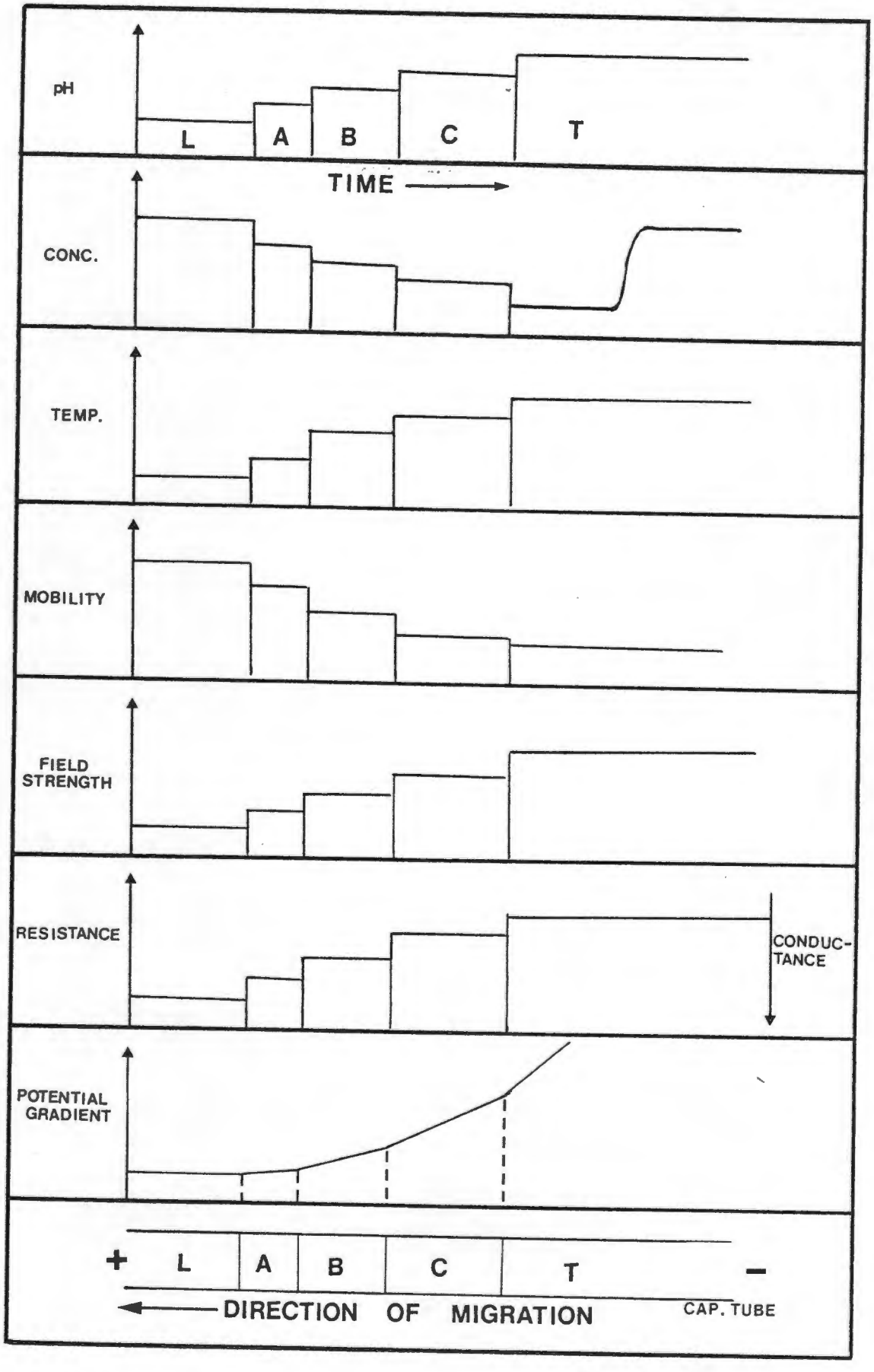
1.3.3.7 Potential Gradient

Separated zones towards the terminating electrolyte are under an increasing potential gradient, thus enabling all zones to move at the same velocity.

All the above parameters are illustrated in Figure 1.4.

FIGURE 1.4

Diagramatic representation of the important properties of the anionic system at equilibrium where the mobility of the leading ion (L) is greater than the sample constituents A, B and C and also the terminating ion (T).


pH
L
A
B
C
T
TIME
CONC.
TEMP.
MOBILITY
FIELD STRENGTH
RESISTANCE
CONDUC-TANCE
POTENTIAL GRADIENT
+
L
A
B
C
T
−
DIRECTION OF MIGRATION
CAP. TUBE

There are two factors that are unique to isotachophoresis. These are the self-sharpening and concentration effects.

1.3.4 Self-Sharpening Effect

The boundary between each separated zone is sharpened under constant current by the electric field which increases towards the terminating electrolyte to compensate for the low mobility ions. Should an ion of low mobility move forwards out of its zone due to diffusion and convection into a region of lower field strength, then the velocity of this ion under the new field strength conditions is decreased. It will, therefore, be overtaken by the leading edge of its own zone. If this ion should move backwards into a zone of higher field strength, its velocity will be increased and thus forced back into its own zone (Everaerts, 1972).

There are a number of factors that influence the self-sharpening effect. These are diffusion constants of the ions, ionic mobility, field strength, temperature differences both longitudinally and transversely across the capillary (Everaerts and Verheggen, 1974; Rýslavý et al., 1977), current density (Everaerts et al., 1976a), leading electrolyte concentration (Arlinger, 1974b), disturbances that result in electroendosmosis, diffusion and convection (Arlinger, 1971, 1974b), viscosity of leading electrolyte and capillary diameter (Verheggen et al., 1977). The self-sharpening effect

actively counteracts mixing due to diffusion and convection (Arlinger, 1971, 1974a).

There is a finite sharpness for each zone boundary under defined experiment of conditions (Arlinger, 1974b).

Some of the above mentioned factors are examined in more detail below.

The zone boundary sharpness is partly dependent on the field strength. The higher the field strength, the sharper the boundary. But the high voltage may lead to excessive heating which will degrade the sharpness.

The sharpness of the zone boundary is decreased if the leading electrolyte concentration is reduced below 0,001 M (Arlinger, 1974b).

The temperature effects on the zone boundary are more pronounced in zones located towards the rear of the capillary. This is due to increased resistance resulting in heating. There is also increased diffusion which degrades the boundary sharpness.

The effect of temperature differences between the centre of the capillary and wall are also more pronounced towards the rear of the capillary, leading to zone boundaries parabolic

in shape (Everaerts and Verheggen, 1974). It is, therefore, necessary to carefully thermostat the capillary tube. This is achieved by mounting the capillary plate on Peltier elements. Heat transfer is aided if small diameter capillary tubes are used. Other factors include placing the capillary in kerosene (paraffin); using metal as the base of the capillary plate; and ensuring the best contact of the capillary plate to the Peltier assembly, using silicone grease.

It is possible to counteract the effects of electroendosmosis by the addition of non-ionic detergents. These remove or reduce the surface charges on the capillary wall. Triton X100 (0,05 - 0,2% v/v) may be added to electrolytes. There is a marked increase in the zone boundary sharpness in aqueous solutions, but Triton X100 does not improve the boundary sharpness in non-aqueous solutions - e.g. methanol and ethanol (Arlinger, 1974b; Everaerts and Verheggen, 1975). Polyvinyl alcohol has the same effect as Triton X100 (Arlinger, 1974b). However, Verheggen et al. (1977) reported increased convective disturbances when 0,05% (v/v) polyvinyl alcohol was added to the leading electrolyte. These surfactants also increase the heat transfer factor and, thereby, reduce the parabolic zone profile (Everaerts and Verheggen, 1974) and reduce convective and diffusion disturbances. The Teflon capillary also reduces the electroendosmotic effects as compared to glass, etc., (LKB Instrument Manual, 1977).

In the separation of high molecular weight substances, where gravitational forces result in instability of the very concentrated separated zones, long chain polymers may be added to the leading electrolyte. The most commonly used polymer is hydroxypropyl methylcellulose (0,5% m/v) (Moberg, 1974; Kjellin et al., 1975a, 1975b; Arlinger, 1975; Delmotte, 1977). Hydroxyethyl cellulose has also been used (Mikkers et al., 1978c). These polymers also reduce electroendosmosis effects (Holloway and Pingoud) 1981).

1.3.5 Concentration Effect

The concentration effect occurs because the low conductivity in a zone of dilute ions results in a high potential gradient. This causes the ions to move at a higher velocity than those in the preceding zone where the voltage gradient is lower. The resultant "heaping up" effect increases the concentration of the sample ions. This, in turn, decreases the electrical resistance and, therefore, field strength. Consequently, the concentration of the sample ions is increased (Figure 1.3). The sample is usually introduced at a concentration less than the leading electrolyte. This also results in an increase in conductivity, a decrease in electrical field strength and, therefore, a decrease in the effective ion velocity.

Using the same logic, if a sample with a concentration greater than the leading ion is introduced into the system, it will be diluted.

The concentration in each separated zone is homogenous; therefore quantitative studies are possible. A large number of ions will give a longer zone and dilute sample ions will be concentrated into a shorter zone.

1.3.6 Influence of Sample Ions on the Leading Electrolyte

In order to conduct an isotachophoretic experiment, it is essential that a leading ion be chosen with a mobility greater than the sample ions. Should the sample contain a component that has a mobility greater than the chosen leading ion, this component will migrate electrophoretically through the leading electrolyte and superimpose its physical properties on the isotachophoretic system. Thus, the isotachophoretic functions of the chosen leading ion are transferred to the more mobile component and the former leading ion now acts as a spacer.

This situation is illustrated in Figure 1.5 where L_1^- in the sample AB has a mobility greater than the chosen leading ion L_2^- and AB. L_1^- migrates through the original leading electrolyte L_2^- and takes over the functions of the leading electrolyte. The fraction of L_2^- that has been overtaken now acts as a spacer.

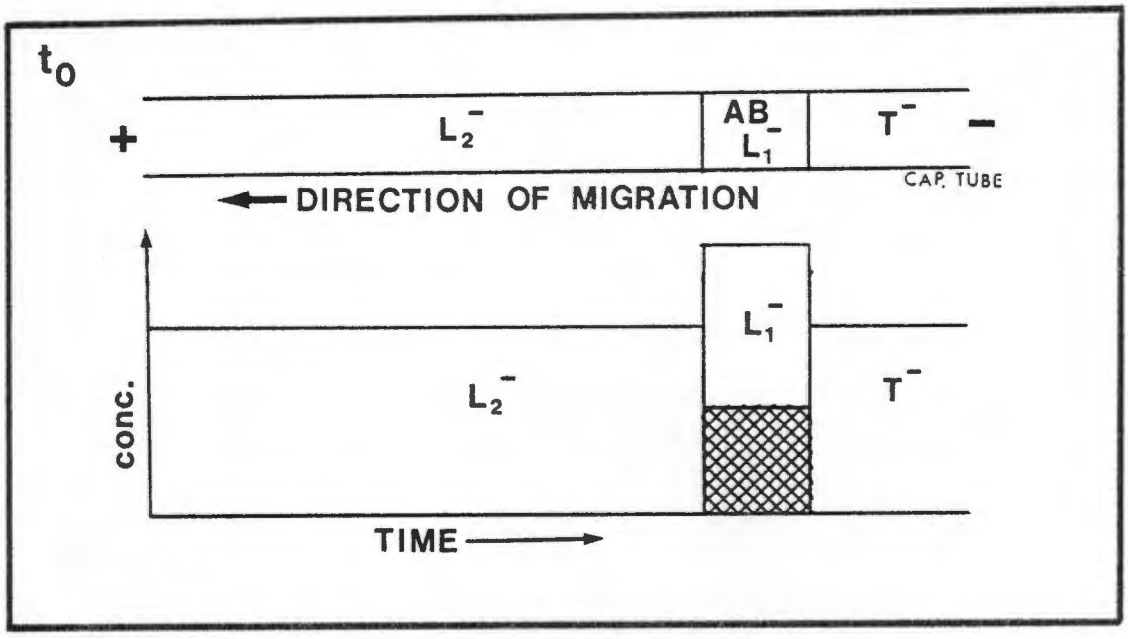
FIGURE 1.5

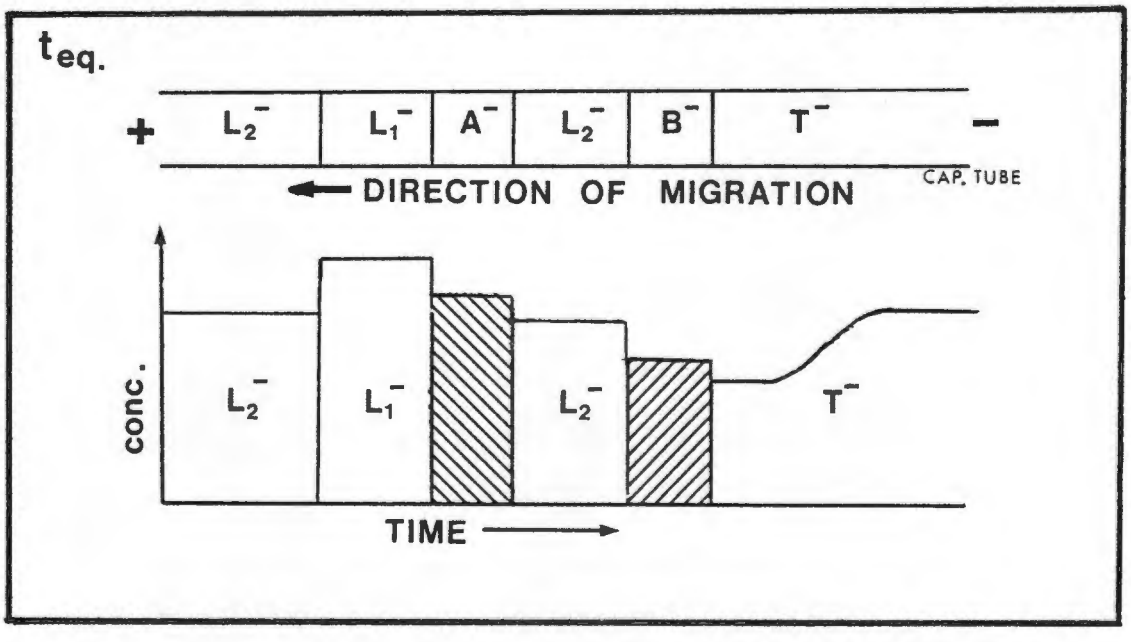
Schematic diagram showing the influence of a sample ion on the isotachophoretic separation of a sample.

t_o Initial conditions in the capillary tube (Cap. tube) where the sample (AB) which also contains a high mobility (m) ion (L_1^-) is introduced between the leading electrolyte (L_2^-) and the terminating electrolyte T^-).

$$m_{L1^-} > m_{L2^-} > m_{AB} > m_{T^-}$$

t_{eq} Conditions at equilibrium where sample AB is regulated by the L_1^- leading ion and the fraction L_2^- that has been overtaken by L_1^- acts as a spacer.


t_0
+
L_2^-
AB
L_1^-
T^-
−
CAP. TUBE
DIRECTION OF MIGRATION
conc.
L_1^-
L_2^-
T^-
TIME


$t_{eq.}$
+
L_2^-
L_1^-
A^-
L_2^-
B^-
T^-
−
CAP. TUBE
DIRECTION OF MIGRATION
conc.
L_2^-
L_1^-
L_2^-
T^-
TIME

1.4 ADVANTAGES AND DISADVANTAGES OF ISOTACHOPHORESIS

There are many advantages in using the closed capillary isotachophoretic system. As a high voltage may be applied to the system (10 - 30 kV), the analysis time is reduced. It may be as little as 4 minutes where ortho- and pyrophosphate in artificial fertilizers was examined (Boček et al., 1978b). But in protein separations, the analysis time is usually 40 - 60 minutes (Delmotte, 1977; Kjellin and Hallander, 1979a, 1979b; Smuts et al., 1982).

Under normal conditions, there are no disturbances to the electrolytes and sample caused by evaporation. Electroendosmosis and hydrodynamic flow, which occur in most other forms of electrophoresis, are reduced (Everaerts et al., 1973).

The absence of a support medium - e.g. polyacrylamide gel or agarose, results in the elimination of sieving effects that cause problems when examining high molecular weight molecules. The variations in physical and chemical properties of the support medium are also eliminated (Delmotte, 1979).

The sample ions, if at a concentration less than the leading ion, are concentrated by this technique. Thus, dilute samples can be successfully examined. Only small sample volumes are required in the analysis (10 μL CSF or 1 μL serum) and this is

advantageous where the sample material is in short supply. Pretreatment of the sample is not required as may be necessary in gas chromatography and high performance liquid chromatography (Holloway and Pingoud, 1981).

The samples need not be both amphoteric and have pI values in the pH range offered by carrier ampholytes in isoelectric focusing. There is also no restriction on the analysis of extremely acidic or basic molecules which have pI values outside the available range of ampholytes (Holloway and Pingoud, 1981).

A wide variety of electrolytes with a large pH range can be chosen for isotachophoretic analyses.

It is possible to increase the resolution of a sample by diluting the leading electrolyte concentration. This results in a dilution of the sample zones and, therefore, longer zone lengths and hence an increase in resolution if the method of detection is sufficiently sensitive (Arlinger, 1974b).

In isotachophoresis, the results of an experiment are immediately available as the detectors form an integral part of the instrument. A variety of detectors are available. These include the high resolution non-specific potential gradient and conductivity detectors. Samples - e.g. proteins which

contain tryptophan and tyrosine may be examined by sensitive UV detectors.

Isotachophoresis may also be useful in pre-separation or pre-concentration of samples which may then be analyzed by liquid chromatography and high performance molecular sieve chromatography (Schoots and Everaerts, 1983; Ofverstedt and Eriksson, 1984).

A few disadvantages exist. The point of injection is critical if reproducible results are required (Everaerts et al., 1976a). This may be overcome by using an injection block (Everaerts and Verheggen, 1975). Once the sample has been separated and detected, it is lost unless it is collected on a moving cellulose acetate strip by means of the Tachofrac. Only one sample can be analyzed at a time but, with the proposed method of isotachophoretic separations on polyacrylamide gels, more samples may be analyzed simultaneously (Holloway and Pingoud, 1981).

1.5 THEORY

The theory of isotachophoresis is based on the regulating function described by Kohlrausch in 1897. The equation expresses the conditions that occur at the boundary between two salt solutions having one ion in common. This boundary is maintained sharply as the ionic species migrate in an electric

field. The solutions are chosen with one containing ions of high mobility (α) and the other slow ions (γ) having the same charge sign.

The concentrations of these ions is expressed as a ratio of their mobilities and is given by

$$\frac{c\alpha \; x\gamma}{c\gamma \; x\alpha} = \frac{z\gamma \; m\alpha \; (m\gamma - m\beta)}{z\alpha \; m\gamma \; (m\alpha - m\beta)}$$

Where c is the molar concentration, x is the percentage degree of dissociation, z is elementary charge and m the mobility of the α, γ and β ions (with β of opposite charge to α and γ and common to both solutions)

If the concentrations differ from those specified by the equation, they will regulate automatically to those required by the regulating function.

The derivation of the Kohlrausch equation is given in Appendix A.

1.6 DISCUSSION

Since the description of the Kohlrausch regulating function in 1897 (Kohlrausch, 1897) and the development of the commercial apparatus in 1974 by LKB-Produkter AB, Sweden, isotachophoresis has been used in the separation of a wide variety of substances ranging from metal ions to proteins.

The sample ions are separated according to their mobilities in a high electric field. At equilibrium, the separated sample ions move with the same constant velocity between the leading and terminating electrolytes.

The choice of a single leading and terminating ion is not a prerequisite for establishing an isotachophoretic steady state. Mikkers and Everaerts (1981) describe two isotachophoretic systems, a terminating frontal system and a leading frontal system, where more than one leading and/or terminating constituent is used to generate multiple moving boundaries with "in stack" and "out of stack" isotachophoretic subconfigurations.

However, once the steady state has been established, a number of parameters may be defined.

There is a temperature change at each zone boundary with an increase towards the terminating electrolyte. The pH generally increases in this direction. The pH of the sample zones may vary from one tenth to several pH units (Everaerts and Routs, 1971). It is, however, possible that the pH in consecutive zones may remain constant. Field strength, voltage gradient and resistance all increase towards the terminating electrolyte. However, the ionic mobility, concentration and conductivity decrease in this direction.

Some of the parameters mentioned above may be used in detecting the separated zones. These are temperature, resistance/conductivity and voltage changes.

The two parameters unique to isotachophoresis are described. The self-sharpening effect of the zone boundaries actively counteracts diffusion and convection. A number of factors which influence the sharpness of the boundary are also given. These include temperature, field strength and concentration of leading electrolyte. Electroendosmosis may also affect the sharpness of the boundary but is counteracted by adding non-ionic detergents to the leading electrolyte.

In the examination of high molecular weight substances - e.g. proteins, the highly concentrated and, therefore, dense zones must be stabilized with long chain polymers - e.g. hydroxypropyl methylcellulose. This increases the viscosity of the solution through which the separated sample zones move and, therefore, electroendosmosis and convection are reduced.

The concentration effect that occurs in isotachophoresis makes quantitative studies possible. The length of the zone is proportional to the number of ions present in the zone. A more detailed review of quantitative studies is given in Chapter V.

Ionic constituents from the sample may superimpose their phys-

ical properties - e.g. higher mobility, on the leading electrolyte and so take over the function of the leading ion. In this manner, the chloride ion present as sodium chloride in the CSF sample has been found to assume the role of the leading ion with the chosen leading ion - i.e. MES acting as a spacer between the FRP and the CSF proteins (See Chapter IV). Although this isotachophoretic regulation may not be very efficient, it has been found to be sufficient to concentrate components.

The advantages and disadvantages of the technique are described. Many of the disadvantages may easily be counteracted. The advantages include short analysis times, small sample volumes and the use of high resolution detectors may be used.

CHAPTER II

DESCRIPTION OF THE APPARATUS AND DETECTION SYSTEMS

2.1 INTRODUCTION

The theory of isotachophoresis was developed and described over 80 years ago by Kohlrausch (1897) and has been discussed in Chapter I. The practical application of the principle has only recently been developed. Everaerts and his co-workers (Martin and Everaerts, 1967; Everaerts and Verheggen, 1970b) in Eindhoven, The Netherlands, first developed the capillary system and it is on their work that the commercial apparatus is based.

Kendall (Kendall and White, 1924; Kendall, 1928) attempted the separation of isotopes in an agar gel column 1000 - 2000 cm long which was submerged in a trough of running tap water. Although the separations were disappointing, he realized the capabilities of the principle, including the importance of the concentrating effect which could be used in obtaining pure samples of rare earth elements that are normally difficult and time-consuming to purify and concentrate. He was later able to separate the rare earth metals yttrium and erbium, and neodynium and praseodynium (Kendall, 1928).

This work was forgotten until the 1960s when Konstantinov et al. (in Haglund, 1970) successfully separated chemicals, isotopes and amino acids in a capillary tube. The separated zones were detected by refractive index changes.

Ornstein (1964) and Davis (1964) originally made use of the concentrating effect of the Kohlrausch principle in their discontinuous polyacrylamide gel electrophoretic technique. Proteins were stacked in very narrow bands in immediate contact with each other, between a leading electrolyte (chloride ion) and terminating electrolyte (glycine ion). Detection of these bands was not possible, so a second analysis step of zone electrophoresis was introduced which further separated the proteins. The concentrating effect is due to isotachophoresis.

The detection of separated zones improved with the development of the thermal (Martin and Everaerts, 1967, 1970), UV (Arlinger and Routs, 1970), conductivity (Everaerts and Verheggen, 1972; Everaerts et al., 1974; Everaerts and Rommers, 1974) and potentiometric (Everaerts et al., 1976a) detectors. Samples of metal ions, amino acids, peptides and complex protein mixtures could now be separated and easily detected.

Analytical isotachophoresis in polyacrylamide gel has been used to separate BSA, bovine haemoglobin (Griffith, 1972; Griffith and Catsimpoolas, 1972; Griffith et al., 1973); immunoglobulins (Ziegler and Köhler, 1976) and subunits of α crystallin (Van Kleef et al., 1977.

Isotachophoresis may also be used as a preparative method in the separation, isolation and concentration of substances. Baumann and Chrambach (1976a) were able to separate gram amounts of the isomers of human growth hormone, bovine serum albumin and ovalbumin. Isotachophoresis in polyacrylamide gel has been used preparatively in the separation of transferrin (Ramsden and Louis, 1971), E. coli enterotoxins (Mölby et al., 1975), histoplasmin (Lancaster and Sprouse, 1977), human serum proteins (Svendsen and Rose, 1970; Routs, 1973; Haglund, 1973; Kopwillem et al., 1975; Hjalmarsson, 1975), and haemoglobin (Haglund, 1970; Svendsen, 1973). However, there are a few problems associated with this preparative method as one is limited by the solubility of the protein, sieving effects, and also heating within the stacked protein zones which results in cracking, deformation and separation of the gel from the column walls (Baumann and Chrambach, 1976a). Wall adherence and mechanical stability of the gel could be improved by substituting N,N'-diallytartardiamide (DATD) for bis acrylamide (Baumann and Chrambach, 1976b).

Resolution in preparative isotachophoresis is optimal when

the steady state has been attained and the steady state decays soon thereafter. The time taken to establish the steady state has no effect on resolution (Houghten and Chrambach, 1977). Ngugen and Chrambach (1981) describe optimum conditions for establishing this steady state.

Bier et al. (1977) used Sephadex columns to prevent convection in their isotachophoretic separation of human serum proteins. This resulted in an improved resolution compared to polyacrylamide gel, and better reproducibility. However, the separation was not as good as anticipated from the theory. A Sephadex G25 column was used successfully to purify retinol-binding protein by isotachophoresis (Ofverstedt et al., 1983).

Preparative isotachophoresis in a flat bed of granulated gel was attempted by Battersby and Holloway (1982) to separate cat liver cystosolic glutathione S-transferase. However, the resolution obtained could not be fully exploited due to technical difficulties associated with sectioning the flat gel. Electroendosmosis and counterflow effects were found to adversely affect resolution in Sephadex G25 gel slabs (Ofverstedt et al., 1981).

Agarose gel has been successfully used in the analysis of human sweat and urine (Uyttendaele et al., 1975, 1977).

Micropreparative capillary isotachophoresis has been described

by a number of workers (Arlinger, 1976; Hjalmarsson, 1977; Kjellin and Siden, 1978). This consists of a fraction collector device, Tachofrac (LKB Produkter, Sweden), which is attached to the Tachophor. The separated sample zones are transferred from the capillary under voltage by a counterflow of the leading electrolyte, onto a moving cellulose acetate strip. Immunological, radioactivity and zymogram techniques may then be applied to detect and identify the collected samples.

2.2 DESCRIPTION OF APPARATUS

The LKB Tachophor 2127 (LKB Produkter AB, Bromma, Sweden) consists of two basic units, the high voltage power supply and the analyzer unit (Figure 2.1).

2.2.1 High Voltage Power Supply

This unit produces a constant DC current up to 500 μA irrespective of the resistance of the solutions in the capillary. The maximum voltage output is 30 kV. It is possible to set either a constant current or constant voltage mode. The constant current mode was selected for all experiments used in this study.

A trip circuit switch is incorporated which turns off the current to the Tachophor when a preset voltage is reached.

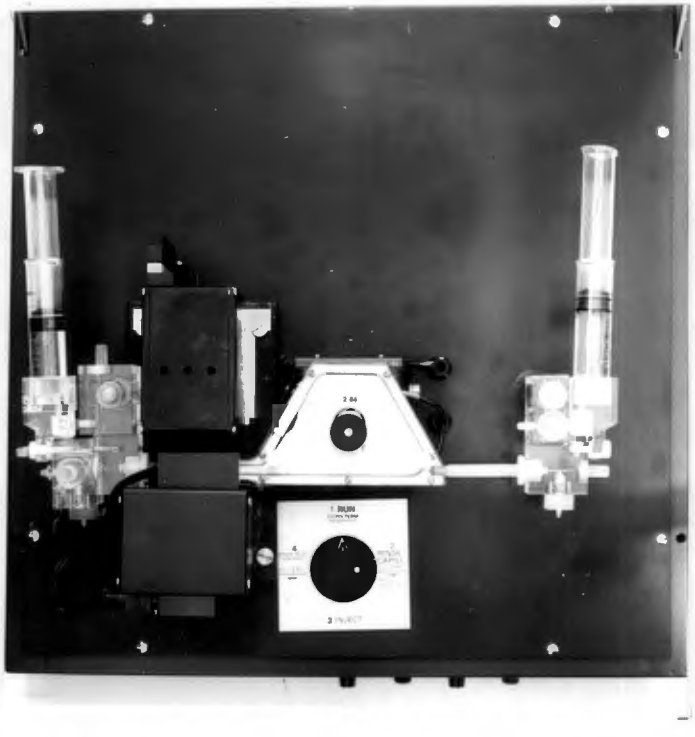
FIGURE 2.1

(a) The LKB power supply, Tachophor analyzer unit, voltage stabilizer and Hewlett Packard reporting integrator.

(b) Detail of the capillary plate, reservoirs and UV detector.

a

b



The polarities of the output may be reversed, allowing either cationic or anionic separations to be performed.

A voltage stablilizer (Langham Thompson, England) was used, together with the Tachophor. This was found necessary as electrical interference from other laboratory equipment affected the recorder signal.

2.2.2 Analyzer Unit

The analyzer unit consists of an electronic and analytical part. There are four printed circuit boards in the electronic part for power supply, capillary temperature control, thermal detection and temperature control of the UV lamp.

The analytical part, under the perspex hood, consists of the capillary plate fitted with a UV and thermal detector, leading and terminating resevoirs, Peltier elements for temperature regulation, high frequency oscillator to excite the mercury lamp and a UV signal detector and amplifier.

2.2.2.1 Capillary Plate

The Teflon capillary, in which the separation occurs, has an internal diameter of approximately 0,5 mm. Capillaries of various lengths are available but, in all experiments described in the present study, the 23 cm capillary was sufficiently long to separate and concentrate the proteins examined.

The capillary is housed in a thermostated plate filled with kerosene. Thermostatic control of the plate is regulated by Peltier elements fitted with heat sink and fan. By changing the direction of the current passing to the Peltier elements, the temperature is either raised or lowered. Two lamps, HEATING or COOLING, indicate whether the system is being heated or cooled.

The sample is introduced through a rubber septum mounted at one end of the capillary. A semi-permeable membrane at the opposite end prevents the hydrodynamic flow of electrolytes.

2.2.2.2 Electrolyte Reservoirs

The perspex electrode vessels are connected to the capillary and each is supplied with a platinum electrode. 20 mL syringes are used to introduce the electrolytes into the reservoirs and capillary via a four-way valve operated by the function knob.

2.2.2.3 Function Knob

Operations to run, rinse capillary, fill terminating and leading reservoirs, inject sample and rinse injection port are controlled by a function knob located on the top of the analyzer unit. This operates the two valves which direct the electrolyte solution to the required sections

of the apparatus. Figure 2.2 is a diagrammatic representation of the capillary plate.

2.2.3 Recorder

A Hewlett-Packard 3390 A Reporting Integrator (Hewlett-Packard, Pennsylvania, U.S.A.), equipped with thermal chart paper, was used to record the separated zones passing the UV detector. The gain control of the Tachophor was set so that at maximum UV absorbance the chart recorder was "in span". The recorder was programmed to determine peak area in integrated units.

The parameters and the settings used were :
baseline position = 0; plot height (ATT 2↑) = 4; chart speed = 6 cm/min; peak width (PK.WD) = 0,04; threshhold (THRSH) = 5; area reject (AR REJ) = 0; INTG ( ) function set at 5 which extends baseline horizontally from last declared baseline point.

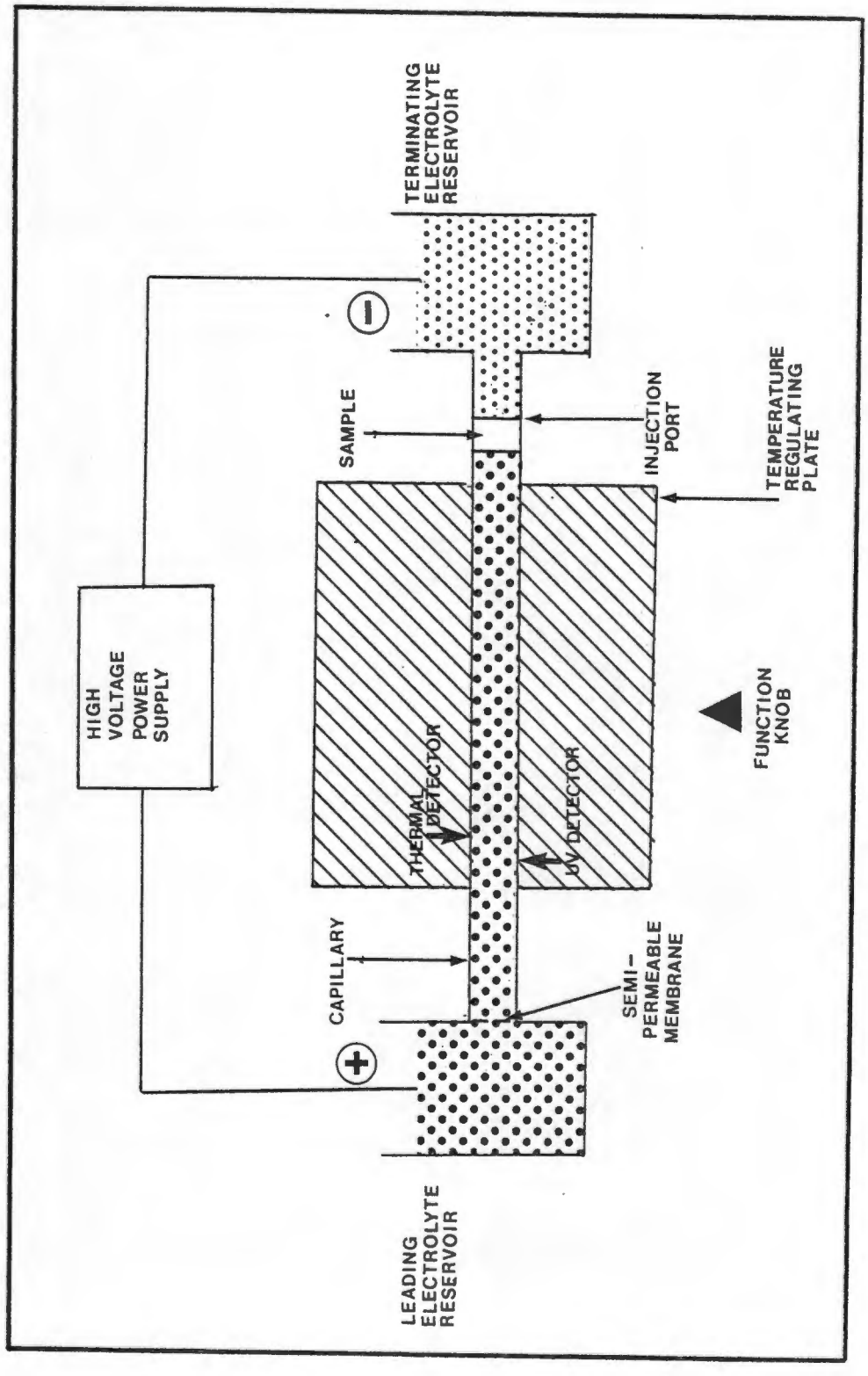
2.2.4 Detection Systems

2.2.4.1 Thermal Detector

In the isotachophoretic system, the separated zones each have different electric fields or potential gradients. Therefore, the heating effect which is proportional to I^2R (where I is current and R resistance), will be different in each zone. Zones with ions of low mobility

FIGURE 2.2

Schematic diagram of the Tachophor.


TERMINATING ELECTROLYTE RESERVOIR
SAMPLE
INJECTION PORT
TEMPERATURE REGULATING PLATE
HIGH VOLTAGE POWER SUPPLY
THERMAL DETECTOR
UV DETECTOR
FUNCTION KNOB
CAPILLARY
SEMI-PERMEABLE MEMBRANE
LEADING ELECTROLYTE RESERVOIR

have a high resistance and, consequently, higher temperatures are produced. There are, thus, consecutive steps of increasing temperature from the leading to the terminating zone.

The thermal detector consists of a tiny thermocouple mounted on the outside of the capillary (LKB Instrument Manual, 1977). The reference for the detector is the thermostated liquid surrounding the capillary. A temperature change on the surface of the capillary is measured as the separated zones pass the sensing thermocouple.

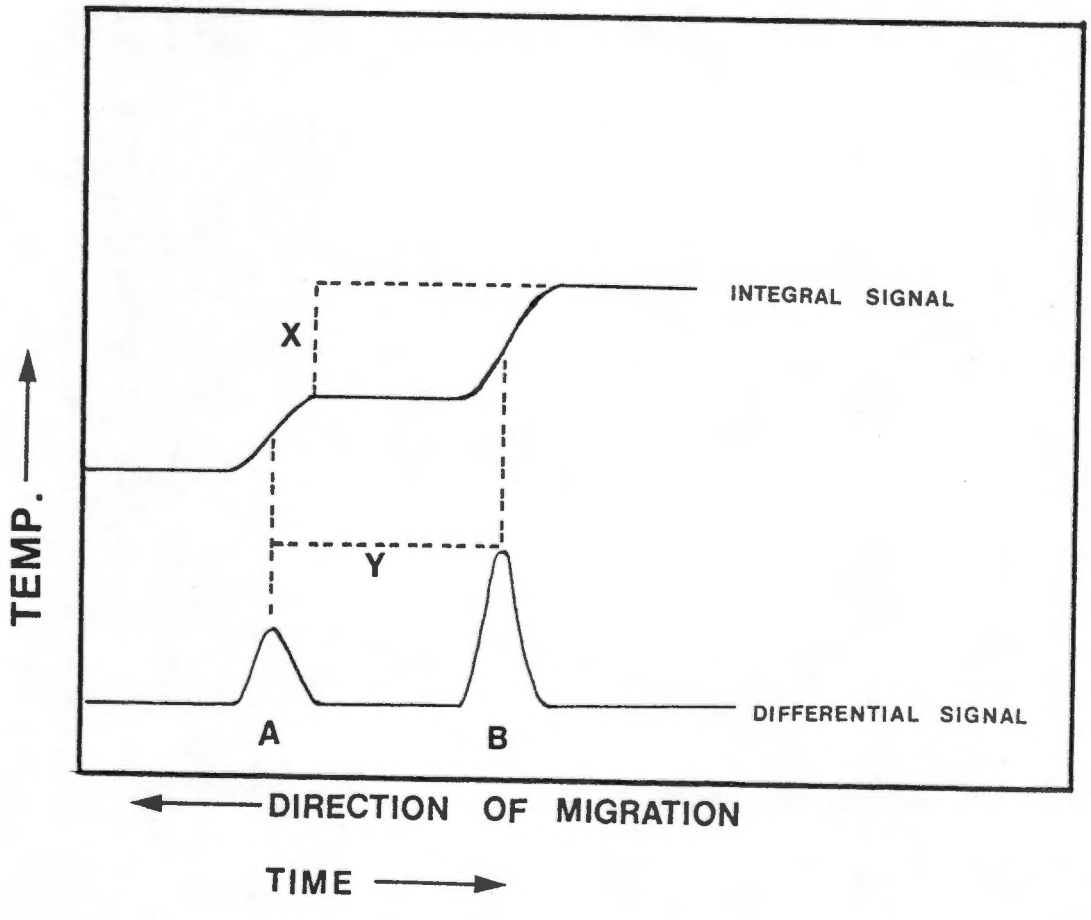
It is possible to obtain both an integral and a differential signal. Figure 2.3 shows the recorded difference between the two signals.

The height of the step (X) (Figure 2.3) of the integral curve represents the temperature increase across the boundary. This is a function of the mobility of the ion species in the corresponding zone and gives qualitative information (Arlinger, 1971).

To measure the zone lengths, the thermal signal is differentiated. The distance (Y) (Figure 2.3) between the two differential peaks is directly proportional to the zone length and concentration of ions in the zone. This

FIGURE 2.3

Diagram illustrating the differences between the differential and integral thermal signal of the thermometric detector.


INTEGRAL SIGNAL
X
Y
TEMP.
DIFFERENTIAL SIGNAL
A
B
DIRECTION OF MIGRATION
TIME

provides quantitative measurements (Haglund, 1970; Everaerts and Verheggen, 1970a; Arlinger, 1971). However, it should be noted that the peaks in the differential curve represent zone boundaries and the peak area has no quantitative meaning (Everaerts and Verheggen, 1970b). Differentiation of the signal may increase the sensititivity of detection (Delmotte, 1979).

Both the integral and differential signals are reproducible (Arlinger, 1971).

The thermal detector is a universal one and is useful in obtaining an overall picture of the characteristics of the zones.

Disadvantages of this detector include a slow response time and a lack of sensitivity compared to other detectors. The minimum zone length that can be detected is 5 mm (Beckers and Everaerts, 1972a). The detector is considered to be 50 times less sensitive than the UV detector (Arlinger, 1974b).

2.2.4.2 UV Detector

The UV detector is located next to the thermal detector in the LKB 2127 Tachophor. It measures the absorbance of material passing a circular aperture with a diameter of 0,2 mm.

The signal from the photo-detector is amplified and then fed to the recorder.

The mercury lamp is excited by a high frequency field created by an HF oscillator. UV light is then emitted. The UV lamp is kept at a constant temperature by a temperature control circuit housed in the electronic part of the analyzer unit. This ensures constant output from the lamp.

The wavelengths most commonly used for detection are 254 nm and 280 nm. However, it is possible to work down to wavelengths of 206 nm. Reijenga et al. (1983a) describe a dual wavelength UV detector with filters of 206 nm, 254 nm, 280 nm and 340 nm. The 206/280 nm combination is especially useful in distinguishing peptides and proteins.

To ensure maximal theoretical resolution, the aperture of the UV detector should be in the range of a few hundredths of a millimetre (Arlinger 1974b). The smallest practical diameter (slit width) is 0,2 mm. However, this may be reduced to 0,1 mm in certain circumstances (Verheggen et al. 1977).

Svoboda and Vacik (1976) have shown that only zone lengths longer than the aperture will be fully detected. A linear relationship exists between the amount of sample

and zone length. If the zone length is less than 0,2 mm, then full UV absorbance height is not reached. There is, however, still a linear relationship between peak height and sample concentration.

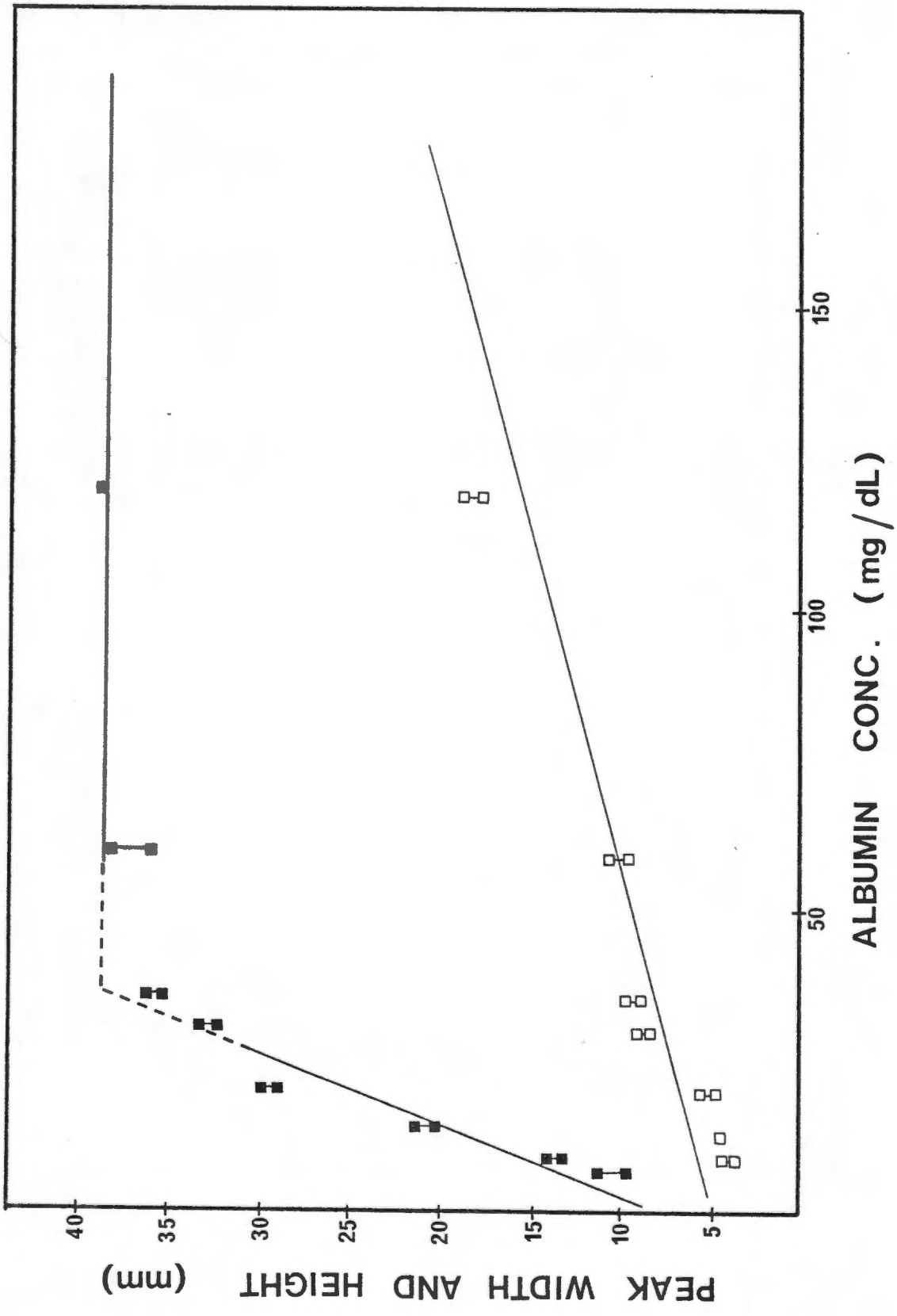
These two characteristics of peak width and peak height are illustrated in Figure 2.4. Varying concentrations of albumin were separated in the MES/Ammediol leading electrolyte system.

The UV detector may also be used in the detection of non-UV absorbing zones. Trace amounts of UV absorbing impurities in the electrolytes and sample may show up as "markers" between major non-UV absorbing zones (Arlinger and Lundin, 1973). As the position of these "markers" is constant under the same separation conditions, it is possible to identify and quantitate the zones bounded by them. The addition of specific UV-absorbing amino acids may also be used to separate two non-UV absorbing zones.

Arlinger and Lundin (1973) have demonstrated the use of UV-absorbing counterions to detect non-UV absorbing compounds. A counterion is chosen with a large difference in molar absorptivity between the acidic and basic forms - e.g. creatinine. The pH difference that exists in the different zones will give rise to absorbance differences

FIGURE 2.4

Relationship between various albumin concentrations and peak width (□—□) and peak height (■—■).


PEAK WIDTH AND HEIGHT (mm)
40
35
30
25
20
15
10
5
50
100
150
ALBUMIN CONC. (mg/dL)

which are detected. The technique is of more importance in the cationic system.

Although UV absorption can give a high degree of resolution, this is partly dependent on the UV-absorbing properties of the sample being analyzed. Adenosine triphosphate (ATP), for example, is a strongly UV-absorbing substance and may be detected to full UV height at 125 pmole (Arlinger, 1974b). Albumin which does not have the same absorbance property can only be detected to full UV height at 10 μM under the anionic conditions of leading electrolyte MES/Ammediol and terminating electrolyte EACA (Figure 2.4). However, it is possible to increase the sensitivity by increasing the recorder amplification.

The advantages of the UV detector include the immediate response and very high sensitivity. Arlinger (1974b) was able to detect a zone length of ATP, 0,1 mm long which corresponded to 16 nL. The UV detector may be used for identification and quantitation of both UV and non-UV absorbing samples (Everaerts et al., 1976a). While the UV detector does not usually disturb the electrophoretic pattern, in some exceptional cases components may be damaged or changed by the UV radiation (Arlinger and Routs, 1970; Everaerts and Rommers, 1974).

2.2.4.3 Conductiometric Detector

Each separated zone in an isotachophoretic system has a defined conductivity (C) and resistance (R) (Figure 2.5). There is an inverse relationship between these two parameters :

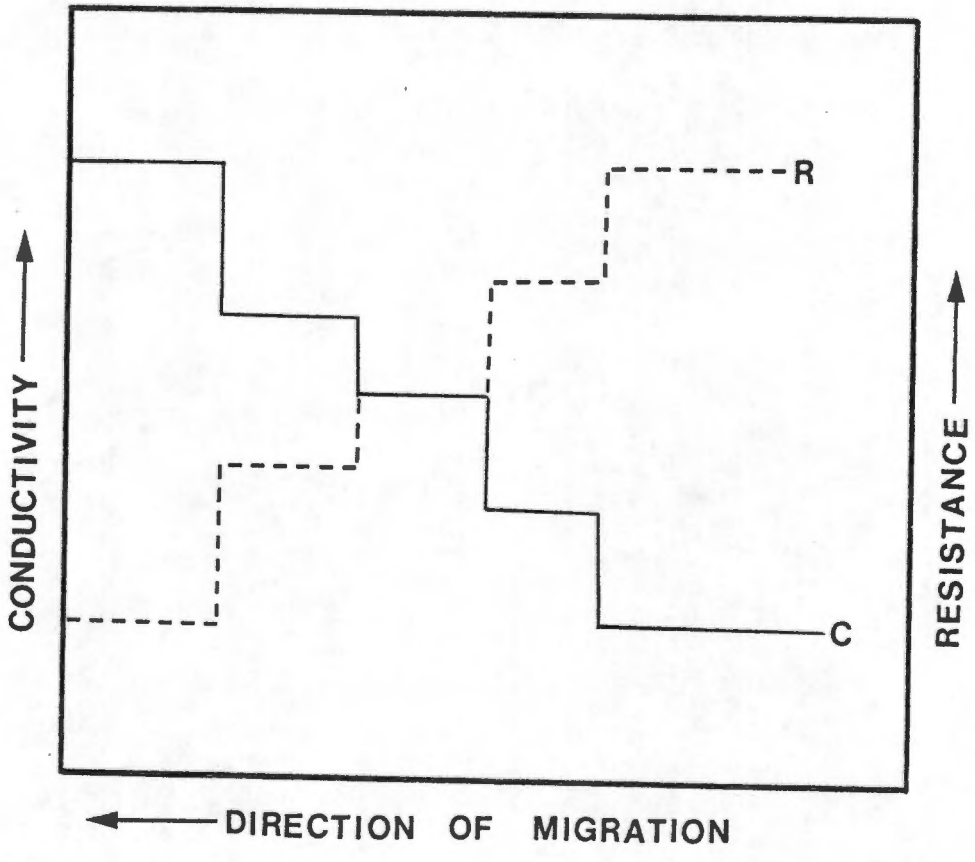
$$C = \frac{1}{R}$$

Everaerts and Verheggen (1972) first developed the conductivity detector. As the 10 µm thick microsensing electrodes of an alloy of platinum and irridium (10% - 30%) were polarized when placed in direct contact with the electrolytes, they were coated with a thin insulating material (Everaerts and Verheggen, 1972; Everaerts et al., 1973; Everaerts et al., 1974; Everaerts and Verheggen, 1975; Everaerts et al., 1976a). The addition of Triton X100 to the leading electrolyte was also found to prevent this polarization which could disturb the measurements (Everaerts and Rommers, 1974).

Kaniansky et al. (1983) describe a simple conductivity detection cell that may be mounted at various intervals along the capillary to monitor separations in aqueous and non-aqueous solutions.

FIGURE 2.5

Schematic diagram showing the inverse relationship between conductivity (C) and resistance (R). Each plateau represents a separated zone.


R
C
CONDUCTIVITY
RESISTANCE
DIRECTION OF MIGRATION

The conductivity detector is universal and may be used for quantitative and qualitative studies. Due to the small time intervals between peaks, quantitative work from tracings on recorder paper has been found to be unsatisfactory (Mulder and Zuska, 1974). It is considered to have the same detection limits as the UV detector (Van der Steen et al., 1972), but Svoboda and Vacik (1976) report that poor results are obtained if small amounts of sample are being separated.

Although there are still a number of practical problems associated with this detector, it has been successfully used in the detection of acids (Everaerts et al., 1973) and uremic metabolites (Mikkers et al., 1979c) and purine and pyrimidine bases which could not be separated with spacers (Oerlemans et al., 1981).

2.2.4.4 Potentiometric Detector

There is a characteristic potential difference between each zone. As this is related to the mobility, the potential differences increase towards the terminating electrolyte.

The voltage increase between each zone may be measured using two noble metal electrodes inserted into the capillary and placed one behind the other (in Hjalmarsson and Baldesten, 1981). Everaerts et al. (1976a) describe an

"AC-converter" which may be used in combination with the conductivity detector to register the voltage gradient.

This detector is as sensitive as the UV detector (Hjalmarsson and Baldesten, 1981) and is also useful in examining non-UV absorbing ions where qualitative information is obtained.

2.3 DISCUSSION

The development of the analytical capillary isotachophoretic apparatus has come a long way from the first experiments of Kendall (Kendall and White, 1924; Kendall, 1928), where long agar gel columns were used in an attempt to separate isotopes.

The first separation to be carried out in a capillary were in 1966 (Konstantinov and Oshurkova, in Haglund, 1970). Use of a capillary resulted in decreased electroendosmotic and convective disturbances. Verheggen et al. (1977) described the importance of the internal diameter (ID) in achieving optimal resolution. From their study, an ID of less than 0,4 mm was preferable with ID 0,2 mm being optimal. At these values, the temperature increase across the capillary is limited and there is stabilization of convection.

The LKB Tachophor is equipped with a capillary ID 0,45 mm (LKB Instrument Manual). In the many studies conducted using this apparatus, resolution is high and the results reproducible.

Electroendosmotic flow may be further reduced by the addition of surface active compounds - e.g. Triton X100. Such compounds have also been found to increase resolution by reducing wall adsorption and diffusion into the wall (Verheggen et al., 1977).

Convective disturbances caused by temperature changes are reduced by adding stabilizers - e.g. long chain polymers of hydroxypropyl methylcellulose (Delmotte, 1977).

Hjertén (1974) reduced convective disturbances by slowly rotating the capillary. In this manner viruses have been successfully separated - e.g. satellite tobacco necrosis virus and also whole cells.

Detection of the separated zones has also been greatly improved. Kendall and White (1924) detected rare earth metals using a hand spectroscope and observing characteristic absorption spectra from the substances. They also successfully separated and detected neodynium and praseodynium by the distinctive

colour of each salt solution. Konstantinov (in Haglund, 1970) used refractive changes to detect separated zones.

The thermal detector was first developed in 1966 (Martin and Everaerts, 1967) and measured relative temperature differences between the separated zones, using a thermocouple fixed to the capillary wall. As the resolution of this universal detector was poor, other highly resolving detection methods were required. In 1972 the conductivity detector was developed (Everaerts and Verheggen, 1972) and later a potential gradient detector (Everaerts et al., 1976a).

The specific UV absorption detector (Arlinger and Routs, 1970) is a high resolution sensitive detector that can accurately measure sharp zone boundaries. It has been successfully used in protein analyses and also for indicating the presence of non-UV absorbing material.

The development of a fraction collector device (Arlinger, 1976) has greatly enhanced the capabilities of capillary isotachophoresis. The capillary system may now be used preparatively to detect, collect and identify the separated samples without losing the resolving power inherent in the method.

The resolving power of isotachophoresis is lost to a certain extent when separation is carried out in polyacrylamide, agarose or Sephadex. However, large scale preparative isotachophoresis has been successfully used (Arlinger, 1975; Baumann and Chrambach, 1976a; Ofverstedt et al., 1983).

With the refinement of detection systems in analytical capillary isotachophoresis and improvement of preparative equipment, the versatility of the technique may be fully exploited. It has been noted that isotachophoresis is being used for the analysis of substances under the zero gravity conditions in space (Bier et al., 1974).

CHAPTER III

MATERIALS AND METHODS

3.1 INTRODUCTION

There is a wide choice of electrolytes that may be used in isotachophoresis. These have been tabulated by Baldesten (1980) and Hjalmarsson and Baldesten (1981). However, for protein separations in an anionic system, only electrolyte solutions of neutral and high pH may be used, so that proteins are all negatively charged. If the cationic system is used, electrolyte solutions of lower pH are required. The choice of the leading and terminating electrolyte solutions is critical for the optimum separation of proteins. For the analysis of all cerebrospinal fluid (CSF) proteins having a wide range of mobilities, it is better to have the leading ion of high mobility and the terminating ion of low mobility. For a more detailed analysis of specific regions in the CSF protein profile, it may be desirable to choose ions of mobilities close to that of the proteins of interest. For immunoglobulin analysis, it may be advantageous to use the cationic isotachophoretic system where all ions in the system carry a net positive charge.

To enhance the detection of proteins at low concentration, the molarity of the leading electrolyte should be kept as low as possible. This will result in longer separated zones which are more readily detected.

Reagents of high quality are essential as impurities in the chemicals may interfere with the separation pattern and affect the interpretation. The use of analytical grade reagents and/or the recrystallization of these chemicals is advisable.

For the analysis of complex high molecular weight components, the use of reagents to increase the viscosity and reduce electroendosmosis are required. Hydroxypropyl methylcellulose (HPMC), hydroxyethylcellulose, Triton X100, polyvinyl alcohol and urea have been used (Baldesten, 1980; Hjalmarsson and Baldesten, 1981). Most satisfactory of these for the separation of proteins is HPMC. As long chain polymers often contain many impurities, extensive dialysis of HPMC against distilled water is suggested by Delmotte (1977). Examples of impurities in the electrolyte pattern caused by HPMC are given in Chapter IV.

Impurities from other sources must also be considered. Fredriksson (1980) noted that impurities from the septum, through which the sample is injected, can cause significant analytical errors. However, this appears to occur only at low

pH. The gradual release of substances adsorbed to the inner wall of the Teflon capillary may also affect subsequent analytical runs and, as a result, it is essential to wash the capillary thoroughly with a non-ionic detergent - e.g. 0,5% (v/v) aqueous solution of Triton X100. In the present study, Triton X100 was left in the capillary overnight. The leading and terminating electrolytes should be prepared daily as organisms may grow in old solutions and produce contaminating substances.

In the analysis of complex protein solutions, the isotachophoretic separation process results in separated components being forced to run in immediate contact with each other. Little information can be derived from such a system. Verstermark (in Hjalmarsson and Baldesten, 1981) reported that the introduction of components of mobility intermediate to those of interest would improve the resolution. These components were named "spacers" and many authors have since made use of these to improve separation (Catsimpoolas and Kenney, 1972; Griffith and Catsimpoolas, 1972; Griffith et al., 1973; Moberg, 1974; Kjellin et al., 1975a, 1975b; Kopwillem et al., 1976; Delmotte, 1977; Kjellin and Siden, 1978; Gallop and Hambleton, 1979; Hedlund et al., 1979; Tourtellotte et al., 1982; Zaffaroni et al., 1983; Del Principe et al., 1985). Both continuous mobility gradients, composed of ampholytes, and discrete spacers of amino acids, were used in this study.

Moberg (1974) notes that it would be advantageous to have a mobility gradient which is independent of pH and only based on the shape, size and charge of the ions. However, there are not enough substances of low mobilities that would create such a gradient and so carrier ampholytes must be used.

It should be noted (more information and experimental data in Chapter IV) that the protein zones are diluted when ampholytes of the same mobility are added and this is seen as a widening of the peak width and a decrease in the peak height.

3.2 MATERIALS AND METHODS

3.2.1 Cerebrospinal Fluid Samples

Cerebrospinal fluid (CSF) samples were stored at -20°C until required. The CSF samples were grouped by clinical and laboratory criteria as normal, acute viral meningitis, subacute sclerosing panencephalitis (SSPE), presumptive multiple sclerosis (MS), Guillian-Barré syndrome (GBS), bacterial meningitis and cryptococcal meningitis. In each case of viral, bacterial and yeast infection, the organisms isolated were identified by standard techniques.

Standard clinical criteria and the demonstration of a raised measles antibody titre in the CSF were used to identify cases of SSPE (Kipps, et al., 1974). In cases of viral

meningitis, mumps, Coxsackie and a variety of enteroviruses, especially ECHO types, were isolated. Diplococcus pneumoniae, Neisseria meningitidis were the causal agents of some of bacterial meningitis cases.

3.2.2 Solutions for Isotachophoresis

With some minor modifications, the composition of the electrolyte solutions used in all experiments were those described by Delmotte (1977). These solutions were made with high quality glass distilled water. Analytical grade chemicals were used and were recrystallized where necessary.

3.2.2.1 Leading Electrolyte Solution

The leading electrolyte solution consisted of 5 mM (2N-morpholino) ethane sulphonic acid (MES) (Sigma Chemical Co., U.S.A.); 10 mM 2 - amino - 2 methyl 1,3 propanediol (Ammediol) (Sigma Chemical Co., U.S.A.) and 0,5% (m/v) HPMC (Aldrich, U.S.A. or Sigma Chemical Co., U.S.A.). Ammediol was recrystallized once from absolute ethanol. A 1% (m/v) solution of HPMC (viscosity 4000 cps) in distilled water was dialyzed for 3 days against distilled water; the water was changed twice daily.

The working leading electrolyte solution was made from a double strength stock solution of 10 mM MES, 20 mM Ammediol, diluted in equal parts with a stock 1% (m/v) HPMC

solution. The final pH of the working solution was 9,1. No adjustment was necessary.

The stock solutions could be stored at 4°C for two weeks without the electrolyte impurity pattern being adversely affected and aliquots used to make up the working solution.

The working solution was degassed prior to use.

3.2.2.2 Terminating Electrolyte Solution

A 5 mM E amino - n - caproic acid (EACA). (H_2N $(CH_2)_5$ COOH) (Sigma Chemical Co., U.S.A.) solution was adjusted to pH 10,6 with a saturated solution of barium hydroxide (Ba $(OH)_2$. 8 H_2O) (BDH Chemicals Ltd., England). This saturated Ba $(OH)_2$ solution was made up in distilled water and filtered before use to remove undissolved Ba $(OH)_2$ and insoluble barium carbonate (Ba CO_3). EACA was recrystallized once from 80% (v/v) ethanol in water. A fresh terminating electrolyte solution was prepared each day. The solution was degassed prior to use.

3.2.2.3 Spacer Solution

The spacer solutions used in all experiments in the present study, unless otherwise stated, were a mixture of ampholytes and amino acids.

The ampholyte spacer solution (Ampholine carrier ampholytes, LKB Produkter AB, Sweden) was composed of equal volumes of 1% (v/v) ampholyte solutions, from stock pH ranges 40% pH 6-8, 40% pH 7-9, and 20% pH 9-11, diluted in distilled water.

The amino acid spacer solution was a mixture of chromatographic quality glycine, valine, leucine and β alanine (Sigma Chemical Co., U.S.A.). Six mg of each was dissolved together in 10 mL distilled water.

Aliquots of these two spacer solutions were stored at -20°C and only thawed when required.

3.2.2.4 Sample Mixture for Isotachophoretic Analyses

Cerebrospinal fluid was examined without prior concentration or dialysis.

For isotachophoretic analyses, 30 µL of unconcentrated CSF was mixed with 4 µL amino acid spacer solution and 0,6 µL ampholyte spacer solution. 10 µL of this mixture was injected into the capillary of the Tachophor giving a final amino acid concentration of 0,32 µg/µL and ampholyte concentration of 0,02% (v/v).

3.2.3 Operation for Analytical Isotachophoresis

The LKB Tachophor and its high voltage power supply was used in all experiments (LKB Produkter AB, Sweden). The ultraviolet detector was equipped with a 280 nm filter. The isotachophoretic separations were carried out in a 23 cm Teflon capillary (ID 0,5 mm) thermostated at 15°C.

A constant voltage regulator (Langham Thompson, England) was used with the Tachophor in order to stabilize the incoming current because other electrical appliances in the laboratory when switched on/off were found to affect the recorder signal.

Separation experiments were started in the constant current mode set at 200 µA. Once the voltage had risen and activated the trip switch set at 10 kV, the current was reduced to 50 µA. The system was left in this state until the end of the experiment. The analysis time was between 30 and 40 minutes, depending on the amount of salts present in the sample.

Before starting a series of experiments, the capillary and electrode vessels were washed with distilled water to remove the 1% Triton X100 solution which was left in the apparatus, when not in use. According to Arlinger (1974c), there is an improved boundary sharpness when a low concentration of Triton X100 is used (0,05 - 0,2% v/v). The effect of the

detergent lasts for several experiments after the detergent is excluded from the leading electrolyte. A rinsing volume of 50 - 75 mL is needed to eliminate this effect. As the Triton X100 left in the capillary is not completely removed by washing with 20 mL distilled water, the very small residual amount might influence the zone sharpness, although it was not specifically added to any of the electrolytes or sample in this study.

Between each series of experiments, the leading and terminating electrode vessels and capillary were washed and replenished with fresh electrolyte solution, thereby eliminating changes in composition of electrolytes due to electrolysis and carbon dioxide absorption.

After examination of infectious material, the apparatus was flushed with 70% (v/v) aqueous solution of isopropyl alcohol (CH_3. CH(OH). CH_3) (Merck A.G. Darmstadt).

A Hewlett-Packard reporting integrator 3390A (Hewlett-Packard, U.S.A.) was connected to the output of the UV detector of the Tachophor. During sample detection, the chart speed was 6 cm/minute. The integrator was programmed so that maximum UV absorbance was "in span" on the thermal recording paper.

3.3 DISCUSSION

Isotachophoresis is a versatile electrophoretic method as a variety of different electrolyte systems can be selected to obtain optimum separation of the sample of interest. The leading electrolyte establishes conditions throughout the system so the selection of the leading ion, the concentration and pH are very important in obtaining good separation.

The leading ion is chosen with a mobility greater than the sample components sample - i.e. CSF proteins. As most serum proteins and thus CSF proteins have a maximum mobility of 8,0 x 10^{-5} cmV^{-1} sec^{-1} (Routs, 1973), the leading ion must be greater than this. The MES or chloride (Cl^-) ion may be used but, as a leading ion of lower mobility will enhance separation, MES was chosen in most cases as the leading ion in preference to chloride ion.

The pH of the leading electrolyte must be selected so that the net mobilities of the sample constituents will not be equal or close to each other. If only small differences (1 - 2%) exist between mobilities, then diffusion occurs at the zone boundaries which negates any sharpness that could be obtained at the boundary (Routs, 1973). The pH must also be such that all proteins of interest are similarly charged and migrate towards the detector. It is preferable that the pH of the leading electrolyte be about 1 pH unit above the highest pI value of the proteins used to obtain good migration (Baldesten, 1980).

In CSF, the gammaglobulins have the highest pI values of the proteins present (pI 6,4 - 8,9) (Stibler, 1978). Thus a leading electrolyte pH of 9,1 will adequately ensure that all proteins are similarly (negatively) charged and thus migrate in the direction of the detector in the anionic system.

The concentration of the leading ion is 5 mM. This concentration ensures that the hydroxyl and hydroxonium ions play a minimal role in current transport (Arlinger, 1971). Below a concentration of 1 mM, these ions also affect migration (Delmotte, 1979). As the concentration of the leading ion determines the concentrations of all other separated zones and this being related to the zone length, the lower the leading ion concentration, the longer the sample zones. Resolution is, therefore, increased. This is desirable when examining dilute complex solutions like CSF. Thus, a balance must be achieved by maximizing zone length without allowing the OH^- and H_3O^+ ions to play an adverse role. Low concentrations may also cause heating problems with resultant convection and destabilization of the separated zones.

The concentration and pH of the terminating electrolyte must be selected so as not to deviate from the Kohlrausch regulating function. If a deviation does exist, then an equlibrium or steady state will not be established. This will result in poor reproducibility and less sharp zone boundaries. The pH

and concentration of the terminating electrolyte should be such that there are as many ionic charges per unit volume as in the leading electrolyte (Baldesten, 1980). Thus, the concentration is usually the same as the leading electrolyte, namely 5 mM. The terminating ion must also have a mobility that is less than any of the sample constituents so that the leading edge of the terminating zone does not overtake any of the samples which would then move zone electrophoretically in an "out of stack' configuration.

As an alkaline electrolyte system has to be chosen for protein separations, carbonate ions are readily formed when the electrolyte is exposed to carbon dioxide in the air. Thus, barium hydroxide was added to the terminating electrolyte to precipitate any carbonate as barium carbonate which would otherwise move electrophoretically through the sample zones.

The counterion which is added to the leading electrolyte must be chemically stable, pure, have a small effective mobility and good buffering capacity (Everaerts et al., 1976a). In an anionic system, the counterion should be chosen according to the following formula:

$$pK_c - 0,5 < pH_L < pK_c + 0,5$$

Where pK_c is that of the counterion and pH is that of the leading electrolyte (Everaerts et al., 1976a). Ammediol (pK 8,8) was used as the counterion in the leading electrolyte

with a pH 9,1. Substituting these figures in the formula (8,8 - 0,5 < 9,1 < 8,8 + 0,5), it may be seen that the rule is obeyed and the selection of Ammediol for the leading electrolyte counterion provides a good buffering capacity.

Hydroxypropyl methylcellulose is added to the leading electrolyte as this diminishes electroendosmosis in the capillary. More importantly, it stabilizes the very concentrated protein zones that are formed during isotachophoresis (Arlinger, 1974a). A drawback of HPMC is the many impurities present in this compound. Thus, extensive dialysis against water is necessary. The impurity pattern of the electrolytes must also be monitored daily. Should the electrolyte impurity pattern be unacceptable (See Chapter IV), the electrolytes are discarded.

The CSF samples were examined unconcentrated which contrasts with Delmotte (1977) and Kjellin et al. (1975a, 1975b), Kjellin and Siden (1978) and Kjellin and Hallander (1979a), where the CSF samples were concentrated ten times by ultrafiltation. In a series of experiments (not reported here), CSF samples were concentrated by a variety of different methods, namely Minicon B15 macrosolute concentrator (Amicon Corp, Danvers, Ma, U.S.A.), vacuum dialysis and lyphogel polyacrylamide gel (Gelman Instrument Co., U.S.A.). It was found that each technique resulted in proteins in different regions of the CSF isotachopherogram being concentrated. As the differ

ent methods of concentration did not give comparable results, and there were protein losses due to adsorption, the CSF samples were examined unconcentrated. Another disadvantage of concentration was the increase in salt content in the CSF which resulted in longer analysis times. According to Everaerts et al. (1976a), the sample to be examined must not have too high a salt concentration or differ significantly in pH from that of the leading electrolyte, as the reproducibility of the technique would be reduced. In the present study, the CSF samples were examined twice to ensure that the results obtained are reproducible.

CSF samples were not dialysed as reported by other authors (Kjellin and Hallander, 1979a; Delmotte, 1977; Kjellin and Hallander, 1982) as, although shorter analysis times are achieved, there is loss of low molecular weight substances - e.g. folic acid and uric acid from CSF.

The addition of spacers to the CSF greatly enhanced the ability to interpret the isotachopherograms. Both a continuous mobility gradient of ampholytes and specific discrete amino acids were used. The drawback of using ampholytes, which can be considered as amino acids with a large range of pK values and mobilities, is that they may separate with a protein zone and, therefore, dilute it. There is also a "quenching" effect that occurs if too much ampholyte is added, as isotachophoresis can no longer take place. The selection of the concentra-

tion and pH range of ampholytes was determined by trial and error.

The optimum concentration of the ampholytes used is largely dependent on the protein concentration of the CSF samples. A balance had to be achieved between examining low protein concentration normal CSF samples and serum contaminated bacterial meningitis CSF samples. In preliminary studies (Chapter IV), the concentration of amino acids and ampholytes selected was considered appropriate for all CSF sample analyses.

The discrete amino acids that are used in isotachophoresis are mainly restricted to the approximately 20 physiological amino acids. These are, therefore, insufficient to cover the whole range of mobilities required by many analyses (Holloway and Pingoud, 1981). These authors suggest that the range could be considerably increased by the inclusion of low molecular weight pure synthetic peptides.

Delmotte (1977) used three amino acids: glycine, valine and β alanine, to separate the gammaglobulin region. Kjellin et al. (1975a, 1975b) and Kjellin and Hallander, (1979), included gamma amino butyric acid, taurine, TES, serine, glutamine and asparagine in their analyses. In the present study, leucine was added to the three amino acids used by Delmotte (1977). This divided the IgG region into three zones.

After consideration of all the above mentioned factors, the electrolyte and spacer conditions were standardized to obtain optimum separation for all unconcentrated CSF samples examined.

CHAPTER IV

FACTORS AFFECTING THE SEPARATION CAPACITY AND RESOLUTION IN ISOTACHOPHORESIS

4.1 INTRODUCTION

Separation in the isotachophoretic or any electrophoretic system can only occur if the constituents to be separated are charged and have different mobilities. The ratio of the effective mobilities in the mixed state must not equal unity (Mikkers et al., 1979a).

$$\frac{m_i}{m_j} \neq 1$$

where m_i and m_j are the effective mobilities of the ionic species i and j

The term separation capacity may be defined as the maximum amount of two ionic species that may be separated completely under given working conditions (Boček et al., 1978a). This term was introduced when mixed zones were observed, due to a number of factors. These are : capillary length; load capacity (either sample volume or sample concentration); counterflow; current; temperature; choice of the leading and terminating ion and pH of the leading and terminating electrolytes.

Resolution (R*) should not be confused with the separation capacity. It is defined as the fractional separated amount of constituent (i) under consideration (Mikkers et al., 1979a):

$$R^* = \frac{\text{Separated amount of i}}{\text{Amount of i}}$$

Resolution is a complex function and, during the separation process, the resolution increases from zero to a maximum value of 1. At zero resolution, no separation has occurred and the sample constituents remain in a mixed state. The resolution rate should be maximized so that the value of unity is reached in the shortest possible time and with the most convenient experimental conditions (Mikkers et al., 1979b).

Some of the factors previously mentioned that may play a role in separation capacity also affect the resolution and include capillary length, current, pH of electrolytes and the load capacity. Also, the ionic mobilities, dissociation constants, molar concentration of the leading electrolyte and analysis time are added factors.

4.1.1 Capillary Length

This factor affects both the separation and resolution of the sample. It is possible to change the column length by replacing the capillary plate with one having a different capillary length. This may be necessary when complex solu-

tions are examined or in solutions where the constituents have similar mobilities.

Under the experimental conditions used in the separation of CSF proteins, a capillary length of 23 cm was found to be adequate for the proteins to separate, concentrate and reach a steady state before the protein zones reached the detector.

4.1.2 Counterflow

It is possible to increase the separation time or equivalent capillary length by using the counterflow technique. This is the hydrodynamic flow of leading electrolyte in the opposite direction to the migration of the sample zones being analyzed (Hjalmarsson and Baldesten, 1981).

This technique is used if the concentrations of the sample ions are too high so that a steady state cannot be reached in the available capillary length. The counterflow technique may also be used if there are large concentration differences in the sample constituents and if they have nearly identical effective mobilities (Everaerts et al., 1976b).

If the counterflow rate is equal to the speed of migration - i.e. 100%, the formation of sharp boundaries is prevented as

many zones remix. Research has shown that a 30% counterflow is the maximum rate that may be applied (Everaerts et al., 1976b; Vacik and Zuska, 1974). Arlinger (1974b) showed that the counterflow technique can greatly improve the separation capacity. It also increases the range of use of a given capillary.

One disadvantage of this method is the long analysis time. This may result in ionic impurities present in the electrolytes interfering and obscuring the separation profile (Hjalmarsson and Baldesten, 1981), and may also cause a decay in the steady state that has been established (Mikkers et al., 1979a). The practical difficulty of precisely controlling the counterflow rate to 10^{-7} to 10^{-8} mL/sec. is an added problem (Everaerts and Verheggen, 1974).

Different methods have been used to perform counterflow experiments and include a microsyringe dosage pump (LKB Instrument Manual, 1977), membrane pump (Everaerts et al., 1976b), and an osmotic pressure pump (Ryšlavý et al., 1978b).

4.1.3 Load Capacity

A given separation compartment or capillary has a limited load capacity. If the load is excessive, partially separa-

ted zones known as mixed zones will be formed (Mikkers et al., 1979b). Thus, the load capacity or mass of sample has a direct bearing on the separation capacity. Resolution may also be adversely affected with too high a load.

Coxon and Binder (1975) have shown that it is possible to increase the amount of sample considerably by using a column with a rectangular cross-section as compared to a circular cross-section column as the volume per unit length available for separation is greater. Verheggen et al. (1977) suggest that, in order to increase the load capacity, especially for preparative isotachophoresis, it would be preferable to mount a series of narrow-bore columns in parallel, rather than increase the inner diameter.

It is also possible to increase the maximum load capacity of an isotachophoretic system using column coupling (Everaerts et al., 1979). This system uses two tubes of different internal diameters with a larger internal diameter in the preseparation tube. Other methods include counterflow, increasing lengths of capillary, "continuous sampling" (Ryslavy et al., 1978a, 1978b), and preseparation in a conical shaped compartment (Everaerts et al., 1979).

If the load capacity is exceeded, many zone boundaries will be detected as the steady state is not reached before the

zones pass the detector. This is due to the presence of mixed zones between pure zones (Everaerts and Verheggen, 1975).

4.1.4 Current

The electric current is not important in the mechanism of separation but it influences the analysis time and resolution (Griffith et al., 1973; Everaerts et al., 1976a). Arlinger (1974b) showed that the separation capacity was not affected in the current range of 50 - 200 μA.

An inverse proportionality exists between the analysis time and the current applied to the system. Thus, the current should be maximized. However, there is a limit to the amount of current that can be applied to the system as excessive heating may occur. This causes parabolic-shaped zone boundaries (Everaerts and Verheggen, 1974), and convection resulting in poorly defined zone boundaries. Thus, resolution is affected. In practice, a compromise must be found between qualitative and quantitative accuracy and current (Mikkers et al., 1979a).

The response time of the detectors is also a limiting factor in using a higher voltage and, hence, higher current. The thermal detector, with a slow response time, will give poor resolution (Arlinger, 1974b) at high current, while the recording of the UV and conductivity detectors will not be

as adversely affected. Thus, the migration rate of the separated zones passing the detector must not exceed the response time of the detector.

It is necessary to balance the effect of current to obtain the shortest possible analysis time without adversely affecting the resolution. This may be achieved by starting a separation run at a high current and then reducing the current before the sample passes the detector. A short time of resolution will also favour a more efficient use of the electric current (Mikkers and Everaerts, 1981).

4.1.5 Temperature

The separation capacity increases with an increase in temperature as the ionic mobilities are increased by 2 to 2.5% per °C (Arlinger, 1974b). Thus, mobility differences between sample constituents are increased and separation is improved.

However, high temperatures have some adverse effects as the formation of parabolic-shaped boundaries is increased (Everaerts and Verheggen, 1974; Coxon and Binder, 1974b, 1975; Hinckley, 1975). Bubbles appear in the capillary system. Diffusion is increased which results in a decay of the sharp zone boundaries.

The capillary must be effectively cooled so that high enough

electric field strengths may be applied to give good resolution without causing too high a temperature that may adversely affect separation. The Peltier elements and cooling fins provide adequate temperature control of the capillary. It is necessary to compromise and keep the thermostat set at the lower temperature ranges (10 - 20°C) as bubble formation is prevented and chemicals are more stable (Arlinger 1974b).

4.1.6 Electrolyte Molarity and pH

Probably the most important parameter regarding the separation capacity and resolution is the choice of electrolytes, their pH and molarity.

When choosing an electrolyte system for isotachophoretic separation, it is necessary to select a pH value for the leading electrolyte so that the sample constituents have maximum differences in the effective mobilities.

It is the degree of dissociation that mainly influences the effective mobility, as it is directly related to the pH. The degree of dissociation (x) and pH are related by the Hasselbach equation:

$$pH = pK + \log\left(\frac{1}{x} - 1\right)$$

where K is the dissociation constant

A change in the pH value of the leading electrolyte will affect the degree of dissociation and, therefore, the mobility of all sample components. This is because, according to the Kohlrausch regulating function, the conditions of the leading electrolyte determine all the parameters in the succeeding zones.

4.1.6.1 Leading Electrolyte

The leading electrolyte should have a number of characteristics, namely an ion of mobility greater than the sample ions (Routs, 1973), a molarity of 0,01 - 0,001 M to prevent excessive heating and the influence of the hydroxonium and hydroxyl ions. These ions contribute considerably to the transport of current if the leading electrolyte has a concentration below 0,001 M (Delmotte, 1979). A pH must be selected to give optimal differences in the effective mobilities of the sample ions (Arlinger, 1974b; LKB Instrument Manual, 1977) and be greater than the pI values of the sample ions so that these all have the same charge sign (Baldesten, 1980). The counterion that must provide a good buffering capacity to the leading and sample ions.

4.1.6.1.1 Effect of pH Value

The pH of the leading electrolyte must be generally chosen at the average pK of the mixture being analyzed (Everaerts et al., 1973). For anion analyses,

the pH should be at least 1-2 pH units below the pK of the leading constituent. This will lead to a rise in pH from one zone to the other, resulting in reasonable differences in effective mobilities which, in turn, leads to an acceptable time of resolution (Mikkers et al. 1979a).

If a pH value of the leading electrolyte is chosen where there is not much difference between the mobilities of two ions (1-2%), diffusion at the zone boundaries will counteract any separation. Resolution of narrow zones will diminish and long separation times will be required (Routs, 1973).

When the pH of the leading electrolyte is close to the pI values of proteins to be separated, small differences in the pI will influence the isotachophoretic separation to a greater extent than if these proteins were separated electrophoretically at a pH value higher or lower than their pI value. This is due to the steep electrophoretic mobility versus pH curves that proteins exhibit in the region of their pI (Griffith et al., 1973).

Vestermark (1970, 1973) has experimentally determined the pH values occurring on both sides of the moving leading/terminating boundary in different electrolyte systems.

It should be noted that changing the pH of the leading electrolyte in special cases may lead to the order of net mobilities of two compounds being reversed (Everaerts and Routs, 1971; Griffith et al., 1973; Mikkers et al., 1979a).

4.1.6.1.2 Effect of Concentration

The concentration of the leading electrolyte is important in determining the concentration of the succeeding zones. If a high leading ion concentration is chosen with the result that the sample zone lengths are very small ($\leq$ 0,05 mm), the interfacial width forms a high percentage of the very short zone and this has an adverse effect on resolution (Mikkers et al., 1979a). The thickness of the isotachophoretic interfaces is of importance in evaluating the maximum resolution of the method (Coxon and Binder, 1974), and the ideal resolution of unity may never be obtained (Mikkers et al., 1979a).

As the concentration of the leading electrolyte is reduced, there is a dilution in the sample zones and, thus, a corresponding increase in the zone length. This leads to increased detectability (Arlinger, 1974b; Griffith et al., 1973). However, Arlinger (1974b) showed that, when the leading elec-

trolyte concentration was reduced to 0,5 mM HCl, the boundary sharpness and thus resolution of ATP was considerably reduced. At these low concentrations, impurities may contaminate the sample and give non-reproducible results.

Lowering the leading ion concentration results in a lower conductivity in the leading electrolyte and, as a result, the field strength is increased and faster separations occur (Griffith et al., 1973).

Boček et al. (1978a) describe a method which employs a concentration cascade of the leading electrolyte to increase the effective length of the capillary. There is a low concentration of the leading electrolyte at the detector, while a high concentration of the leading electrolyte exists near the sample. All the sample zones adjust to this high concentration and the separated zones are short and migrate slowly. The high and low concentration leading electrolyte boundary is stationary. After the zones pass this boundary they adjust to the lower concentration, become longer and their velocity increases.

4.1.6.2 Terminating Electrolyte

Although the terminating electrolyte does not directly influence resolution and the separation capacity, its concentration and pH must be selected so that it does not deviate from the equilibrium values set by the Kohlrausch formula (Everaerts et al., 1976a; Baldesten, 1980). If the concentration deviates greatly from the leading electrolyte, the sample zones will not reach a steady state before they pass the detector. This will result in less sharp zone boundaries and poor reproducibility (Baldesten, 1980).

It is possible to change the mobility interval between the leading and terminating ion to a limited extent by selecting another terminating ion. A narrow mobility interval avoids large temperature and pH steps between the leading ion and succeeding zones (Catsimpoolas and Kenney, 1972; Griffith et al., 1973).

Where high pH values for the terminating electrolyte are required, the pH is usually adjusted with barium hydroxide ($Ba(OH)_2$) which precipitates any carbonate ions that form when the electrolyte comes into contact with carbon dioxide in the air. The carbonate ions will otherwise move zone electrophoretically through the sample zones

and disturb the zone boundaries (Routs, 1973; LKB Instrument Manual, 1977).

4.1.6.3 Counterion

The choice of the counterion is of importance to ensure that there is maximal buffering capacity at the pH of the leading electrolyte, so that maximum resolution and increased efficiency of separation may be achieved (Mikkers et al., 1979a). If the counterion is used at a pH value well below its pK value, it behaves like a strongly ionic species and, therefore, provides no buffering capacity. The counterion should, thus, be chosen with a pK value no less or greater than 0,5 units above or below the pH of the leading electrolyte (Everaerts et al., 1976a; Holloway and Pingoud, 1981).

The efficiency of current transport is directly influenced by the mobility of the counterion (Mikkers et al., 1979a). Thus, a counterion with a low ionic mobility should be chosen. This will then favourably influence the time of resolution, time of detection and load capacity (Mikkers et al., 1979a) and ensure efficient use of the power supplied to the isotachophoretic system (Mikkers et al., 1979b).

The mobility of the counterion has only a marginal influence on the separation efficiency Mikkers et al., 1979b).

The counterion should also not absorb in the UV region if the UV detector is to be used.

There are only a few substances which satisfy all the requirements of low mobility, good buffering capacity at a suitable pH and no UV absorption (Mikkers et al., 1979a), chemical stability and purity (Everaerts et al., 1976a). Examples of substances that may be used are Ammediol, Tris, creatinine and histidine.

4.1.6.4 Nonionic Detergents and Stabilizers

Boundary sharpness and, thus, resolution is improved with the addition of non-ionic detergents to the electrolytes. The addition of 0,05 to 0,2% (v/v) Triton X100 increased the sharpness of zones as detected by the UV detector (Arlinger 1974b). Polyvinyl alcohol, 0,2 to 0,5% (v/v), may have the same influence as Triton X100 (Baldesten, 1980).

The action of these detergents is to increase the viscosity in the vicinity of the capillary wall and thereby

reduce electroendosmosis (Arlinger,1974b; Everaerts and Verheggen, 1975).

In the separation of high molecular weight substances - e.g. proteins, it is necessary to increase the viscosity of the leading electrolyte so that the highly concentrated zones may be stabilized (Arlinger, 1971). Hydroxyethylcellulose (Everaerts and Verheggen, 1970b) and hydroxypropyl methylcellulose have been used in concentrations varying from 0,5 - 2% (m/v) (Kjellin et al., 1975a, 1975b; Delmotte, 1977; Hedlund et al., 1979). The addition of these long chain polymers also reduces electroendosmosis and so enhances boundary sharpness.

4.1.6.5 Non-Aqueous and Aqueous Electrolyte Solutions

Solvents other than water may be required in order to obtain complete separation of some components. A non-aqueous electrolyte system is usually chosen if the sample species have the same effective mobilities and pK values and/or if they are only slightly soluble in water - e.g. fatty acids, halides (Hjalmarsson and Baldesten, 1981), metal ions (Everaerts et al., 1973). If the hydroxyl and hydroxonium ions interfere with the separation process, a non-aqueous system may also be chosen. The methanol and ethanol systems are most commonly used al-

though Hjalmarsson and Baldesten (1981) report the use of acetone as a solvent.

The addition of a few percent of methanol or ethanol to an aqueous solution may be sufficient to change the mobility of the constituents and so aid in the separation process (Baldesten, 1980). Martin and Hampson (1975) separated insulin in a 70+30 ethanol/water system but found that their results were not reproducible.

The use of aqueous solutions has a number of advantages. These include the ease with which one can adjust the pH of the leading electrolyte to change the degree of dissociation. Water is a good medium for the additives needed to protect the sample from oxidation and reduction, as well as interactions with other samples (Hjalmarsson and Baldesten, 1981); one can add urea (up to 6 M), dithiothreitol, mercaptoethanol and non-ionic detergents to protect the sample (Baldesten, 1980), provided these do not dissociate at the pH used in the separation process (Hjalmarsson and Baldesten, 1981).

Baldesten (1980) has described methods for selecting the electrolytes required for separation. A number of computer programmes have also been written. The Isogen system (Everaerts and Routs, 1971) gives the best electrolytes

and pH value required for separation, provided the mobilities and pK values of the sample ions are known. Jovin (1973) developed the theory generally known as "multiphasic zone electrophoresis" (MZE). Over 4000 electrolyte systems have been generated, of which 1616 are unique as different combinations of leading, terminating and counterions are used.

4.1.7 Sample pH and Concentration

The separation process of isotachophoresis is to some extent dependent on the pH of the sample, but there is little freedom to change this parameter (Everaerts et al., 1976a; Mikkers et al., 1979a, 1979b). However, where the difference in dissociation constants is large, the pH of the sample may be important in the separation process (Mikkers et al., 1979b). In anionic separations, a low pH of the sample favours resolution, provided the transference numbers of the ionic species in the mixed state are independent of pH (Mikkers and Everaerts, 1981). In the present study of CSF proteins, the pH of the sample introduced into the capillary system was pH 8,8.

The concentration of the sample is also of importance in the separation capacity of the instrument. If high concentrations are injected, some of the sample ions are partially lost (Everaerts et al., 1976a) and mixed zones remain for a longer period (Everaerts and Verheggen, 1975). Due to the presence of mixed zones, more zone boundaries will be detec-

ted (Everaerts and Verheggen, 1975). Irreproducible results are also obtained (Everaerts et al., 1976a).

It is thus best to adjust the concentration of the sample to approximately that of the leading electrolyte, but this is not possible for protein analysis.

4.1.7.1 Effect of Ions in Sample on Separation and Resolution

It is possible that ions from the sample may take over the role of the chosen leading ion in the leading electrolyte. This has been found to occur in the examination of CSF samples where the chloride ion present as sodium chloride assumes the role of the leading ion and MES, the chosen leading ion, acts as a spacer between the FRP and the main protein region (manuscript in preparation) (See Page 100).

4.1.8 Amino Acid and Ampholyte Spacers

In isotachophoresis, resolved sample zones are in direct contact with each other, making detection difficult, especially where complex protein mixtures - e.g. serum and CSF, are involved. Separation and resolution may be improved by the addition of substances of intermediate mobilities termed spacers (Vestermark in Haglund, 1970). These may be discrete amino acid spacers or a continuous mobility gradient of ampholytes.

4.1.8.1 Amino Acid Spacers

A discrete spacer is a single compound chosen to have a mobility intermediate to two sample components of interest. Amino acids are chosen and are usually restricted to the common physiological amino acids. They are usually non-UV absorbing and include glycine, valine, leucine β alanine, asparagine, arginine and threonine (Kopwillem et al., 1976; Delmotte, 1977; Kjellin and Hallander, 1979a; Smuts et al., 1982). UV-absorbing amino acids may also be used (Hjalmarsson and Baldesten, 1981). The amino acids not only serve as spacers, but also act as defined mobility markers, making interpretation of an isotachopherogram easier.

As there are insufficient amino acids to cover the whole range of mobilities, ampholytes have to be used in conjunction with the discrete spacers.

4.1.8.2 Ampholyte Spacers

Carrier ampholytes may be regarded as complex mixtures of polyaminopolycarboxylic acids with a wide range of pK values and mobilities distributed over a certain pH range. A mobility gradient is thus created and when a protein sample is added the ion species in the gradient will space the protein zones (Moberg, 1974). These ampholytes are also arranged in order of acidity and a moving pH gradient is created. Thus, proteins in this

moving gradient will separate mostly in order of their pI values. Denaturation and precipitation of the proteins at their pI is overcome by the choice of a counterion which buffers the system in such a way that the resulting pH creates sufficient charges on the ion species of interest (Haglund, 1970).

As the ampholytes may sometimes have the same mobility values as the proteins, these zones may be diluted with the ampholytes. There is thus a widening of the zone with the usual square shape of the UV absorbing zone having a lower maximal amplitude and increased half width (Moberg, 1974; Arlinger, 1975; Svoboda et al., 1983).

The pH range of the ampholytes should be chosen in such a way as to include the mobility range of the proteins in the sample. However, if impurities from the proteins are to be excluded, then a pH range higher or lower than the pI of the protein should be used (Griffith et al., 1973).

Ampholytes are usually used where the protein mixture is very heterogeneous and high resolution is essential (Svendson and Rose, 1970; Arlinger and Routs, 1970; Moberg, 1974; Arlinger, 1975; Delmotte, 1977; Everaerts et al., 1976b).

It would be advantageous to have a mobility gradient independent of pH, spacing the same net mobility range regardless of the pH of the leading electrolyte. However, it is not possible to obtain a large number of ions with a wide distribution of mobilities that are solely based on the size, shape and charge of the ions (Arlinger, 1975).

In the following experiments, the effect of the choice of the leading electrolyte, the choice of amino acids, the pH and concentration of the ampholyte spacers, sample volume and current, on the CSF separation pattern will be examined. The effect of NaCl in the sample on the separation of FRP is also given.

4.2 METHODS AND RESULTS

4.2.1 Sample Volume

4.2.1.1 Method

Different volumes of unconcentrated CSF and spacers were injected into the 23 cm capillary in order to determine the maximum volume or load capacity of the system. The leading and terminating electrolytes were prepared as described in Chapter III.

4.2.1.2 Results

Increasing the volume of CSF injected into the system from 2,5 µL to 30 µL resulted in the presence of mixed zones (Figure 4.1). These are noticeable in the region after the albumin peak and are indicated by arrows. From the curves, it would appear that mixed zones occur when more than 20 µL is injected.

The analysis time is also increased, due to the presence of more salt in the system.

A straight line relationship exists between the amount injected and the area of the peaks provided there are no mixed zones (See Figure 5.10 - Chapter V).

4.2.2 Current

4.2.2.1 Method

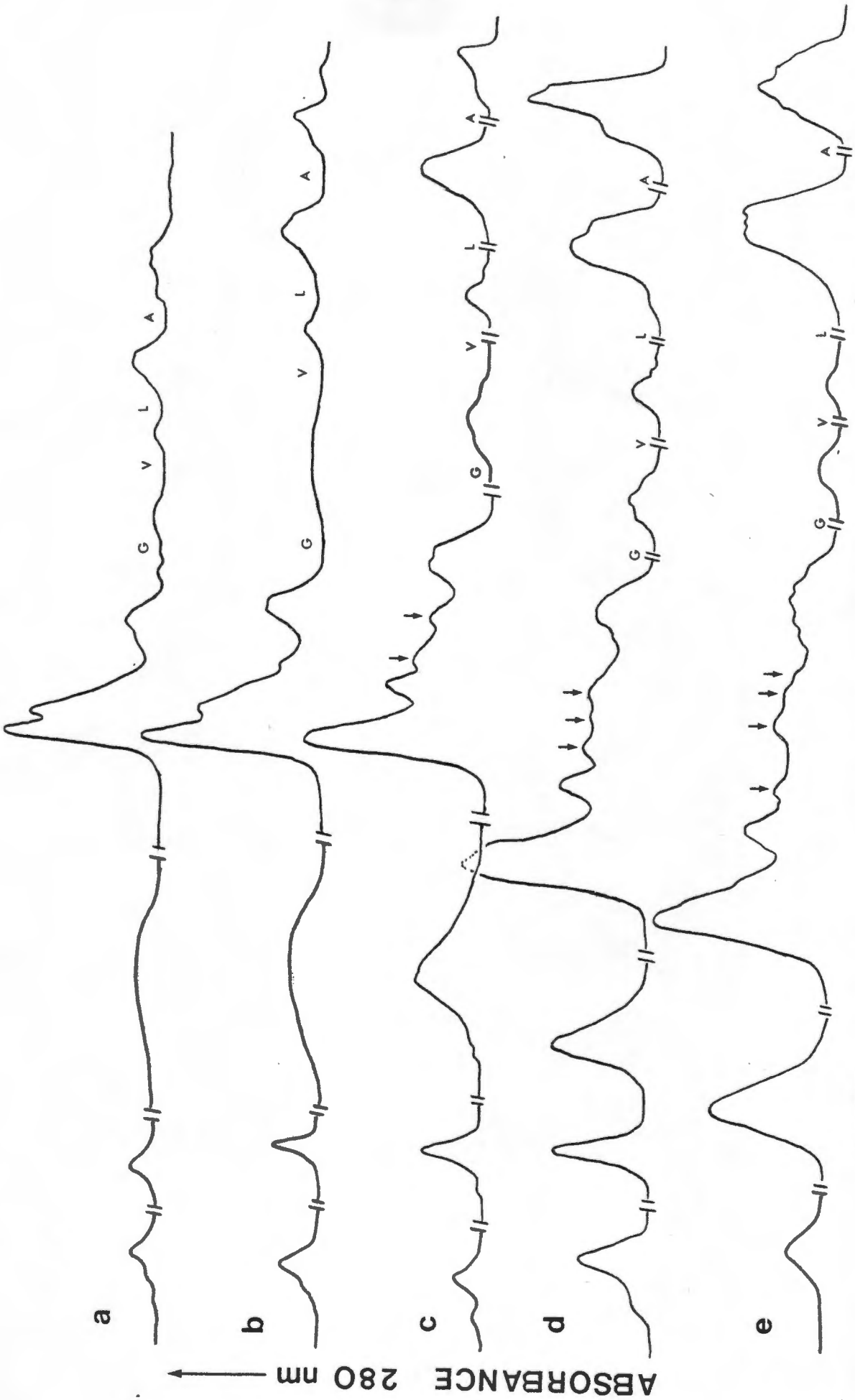
Various current settings were used in the separation of 10 µL CSF with spacers. The chart speed remained constant at 6 cm/minute.

4.2.2.2 Results

With low current, 50 µA, the analysis time for the separation of the CSF sample was 90 minutes. This isotachophoretic pattern was similar to that obtained when the separation was initially started at 200 µA and subsequen-

FIGURE 4.1

Isotachopherograms showing the effect of increasing CSF volume on the separation profile - (a) 2,5 μL; (b) 5 μL; (c) 10 μL; (d) 20 μL; (e) 30 μL.


a
b
c
d
e
G
V
L
A
ABSORBANCE 280 nm
TIME
30 seconds

tly reduced to 50 µA when the voltage had reached 10 kV (Figure 4.2). The analysis time is reduced to 45 minutes. Thus, without losing resolution, it is possible to decrease the analysis time by starting in a higher current mode. More experiments may be carried out in one day as a result.

At 200 µA and 100 µA constant current, the analysis times were 31 and 60 minutes, respectively. An inverse proportionality exists between analysis time and current (Figure 4.3).

The response time of the detector is seen to be a limiting factor as the resolution at 200 µA is less than at 200 µA reduced to 50 µA.

4.2.3 Electrolytes

4.2.3.1 Method

The separation of CSF proteins in the anodic isotachophoretic system requires the pH of both the leading and terminating electrolytes to be high so that all proteins, including the gammaglobulins, have the same negative charge sign.

The leading electrolyte was 5 mM MES, 10 mM Ammediol 0,5% (m/v) HPMC pH 9,1 (Delmotte, 1977).

FIGURE 4.2

Isotachopherograms showing the effect of changing the current on the CSF separation profile - (a) 200 μA; (b) 100 μA; (c) 50 μA; (d) 200 μA reduced to 50 μA when voltage had risen to 10 kV.

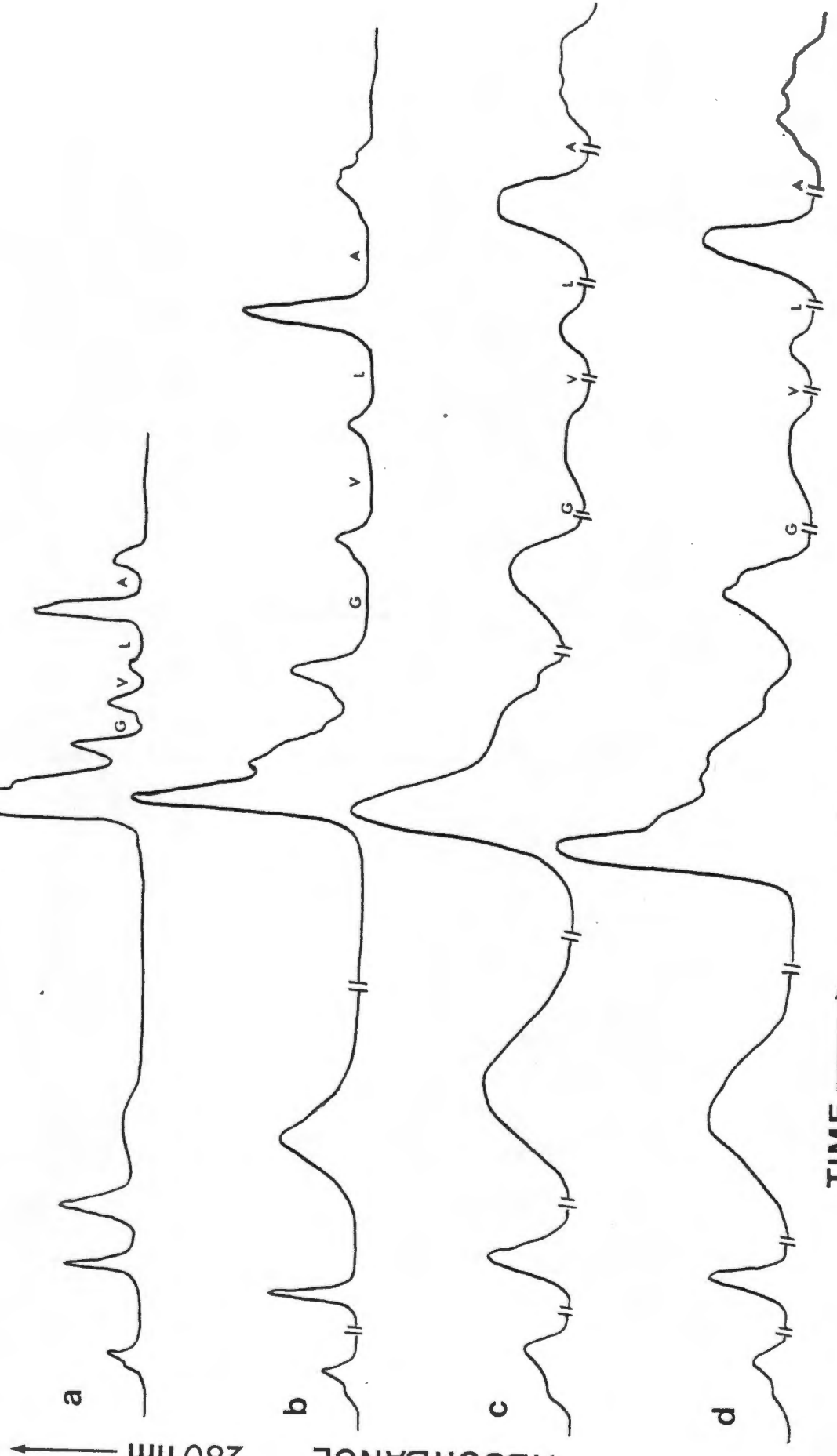
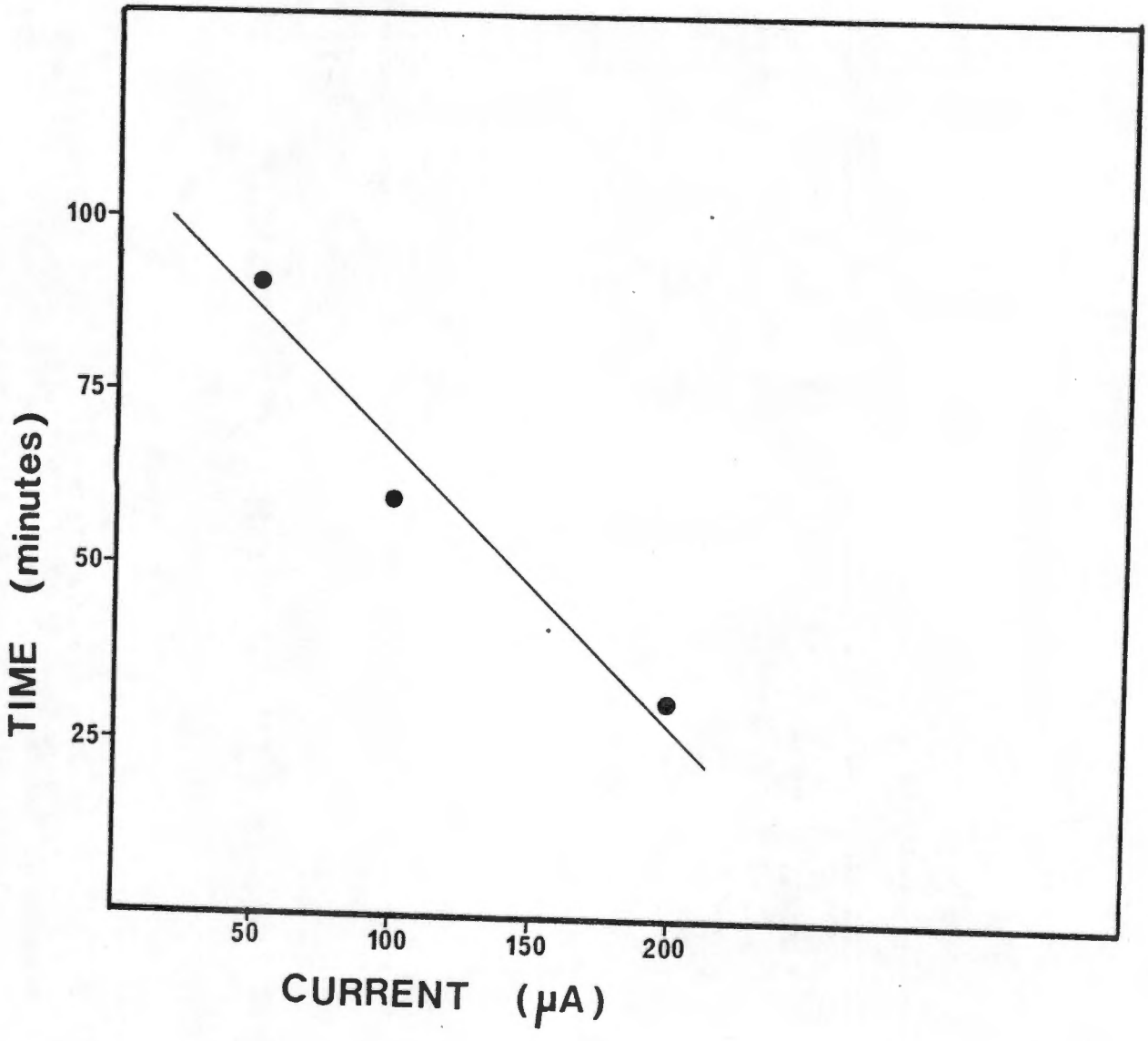

a
b
c
d
G
V
L
A
280 nm
ABSORBANCE
TIME
30 seconds

FIGURE 4.3

Relationship between analysis time and current.


100
75
50
25
TIME (minutes)
50
100
150
200
CURRENT (μA)

The terminating electrolyte was 5 mM EACA, adjusted to pH 10,6 with $Ba(OH)_2$ (Delmotte, 1977).

The effect of a higher mobility leading ion on the separation pattern was examined.

A 10 mM stock HCl (BDH Chemicals Ltd., England) solution in distilled water was diluted 1:2 with a 1% (m/v) HPMC solution. The pH of this mixture was approximately 3. Solid Ammediol was gradually added to the solution until the pH was 9,1.

4.2.3.2 Results

4.2.3.2.1 Electrolyte Impurity Patterns

The electrolyte impurity pattern (EIP) must be examined for each new batch of electrolytes as impurities may interfere with the CSF separation pattern, especially in the gammaglobulin region. The EIP of the MES/Ammediol leading electrolyte system is given in Figure 4.4. The first set of peaks are due to impurities in the electrolytes, while the second set of peaks are from HPMC impurities. The effect of the addition of spacers to the electrolytes results is an almost horizontal baseline.

FIGURE 4.4

Electrolyte impurity patterns (EIP).

(a) EIP of MES/Ammediol leading electrolyte with no HPMC.

(b) An acceptable EIP of MES/Ammediol leading electrolyte with HPMC.

(c) An unacceptable EIP of MES/Ammediol leading electrolyte with HPMC where the chemicals were not recrystallized and the HPMC was not dialyzed against distilled water.

(d) EIP with spacers - i.e. 0,4 µL stock amino acid solution and 0,6 µL stock ampholyte solution.

(e) EIP of HCl/Ammediol with HPMC.

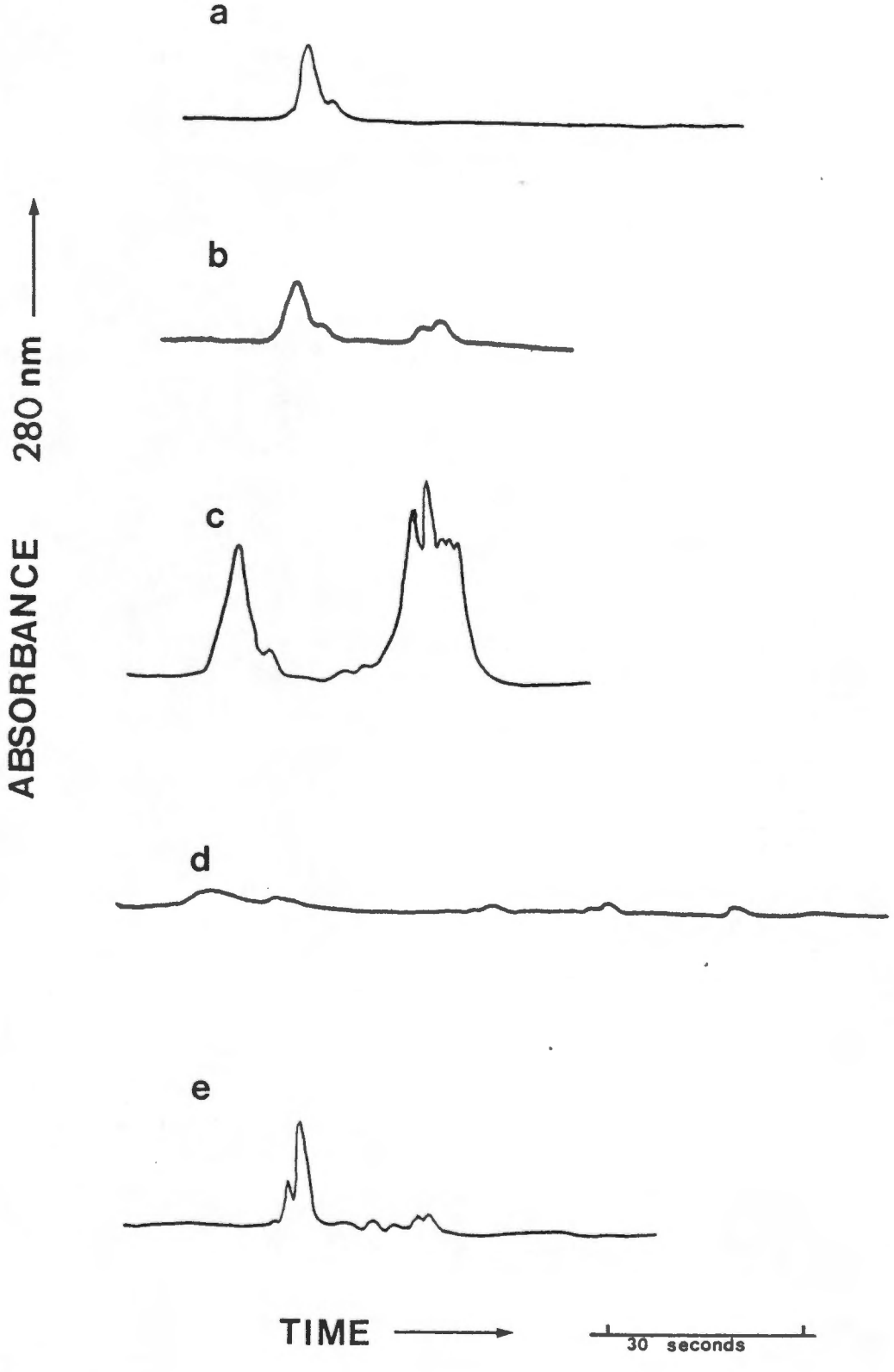

a
b
c
d
e
ABSORBANCE 280 nm
TIME
30 seconds

Figure 4.5 shows the effect of a poor EIP on the CSF separation pattern. The appearance of contaminating peaks in the gammaglobulin region is demonstrated.

The EIP of the HCl/Ammediol system is given in Figure 4.4

4.2.3.2.2 Effect of MES and Chloride Leading Ions on CSF Separation Profile

The effect of HCl/Ammediol leading electrolyte on the CSF profile is clearly shown in Figure 4.6. The position of the front running peaks (FRP) is altered, so that they occupy a position immediately ahead and in contact with the albumin peak. The time difference between the FRP3 and albumin peak is changed from 2,56 minutes to 0,1 minute.

It appears that in the MES/Ammediol system, the FRP move ahead of the leading ion as their mobility is greater than MES. These substances may thus be moving zone electrophoretically in an "out of stack" configuration in the leading electrolyte. (However, see section 4.2.4).

When the chloride ion with a higher mobility is substituted for the MES ion, the FRP are forced into a position immediately behind the chloride and thus

FIGURE 4.5

Isotachopherograms of two CSF samples showing the position of impurities from a poor EIP in the CSF protein profile.

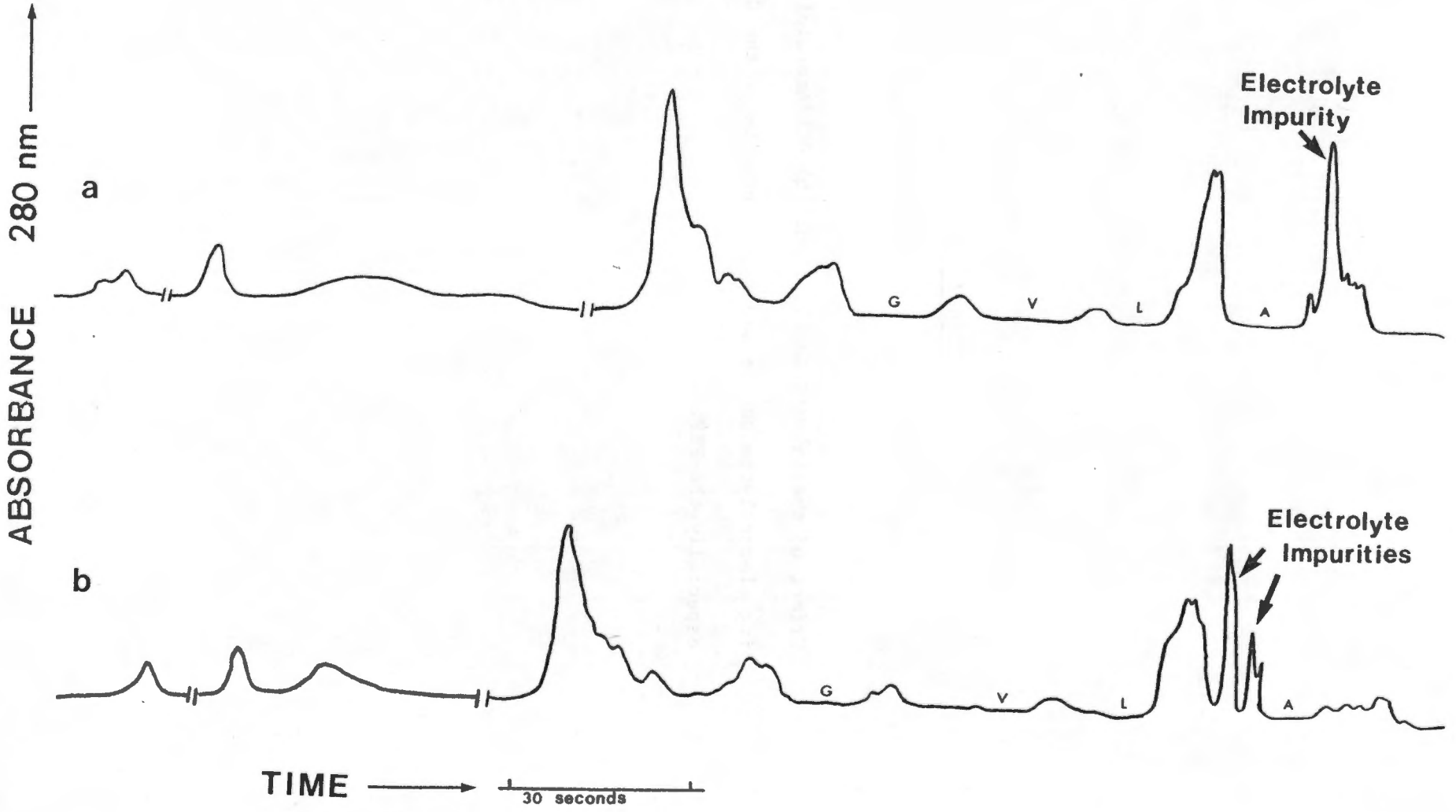
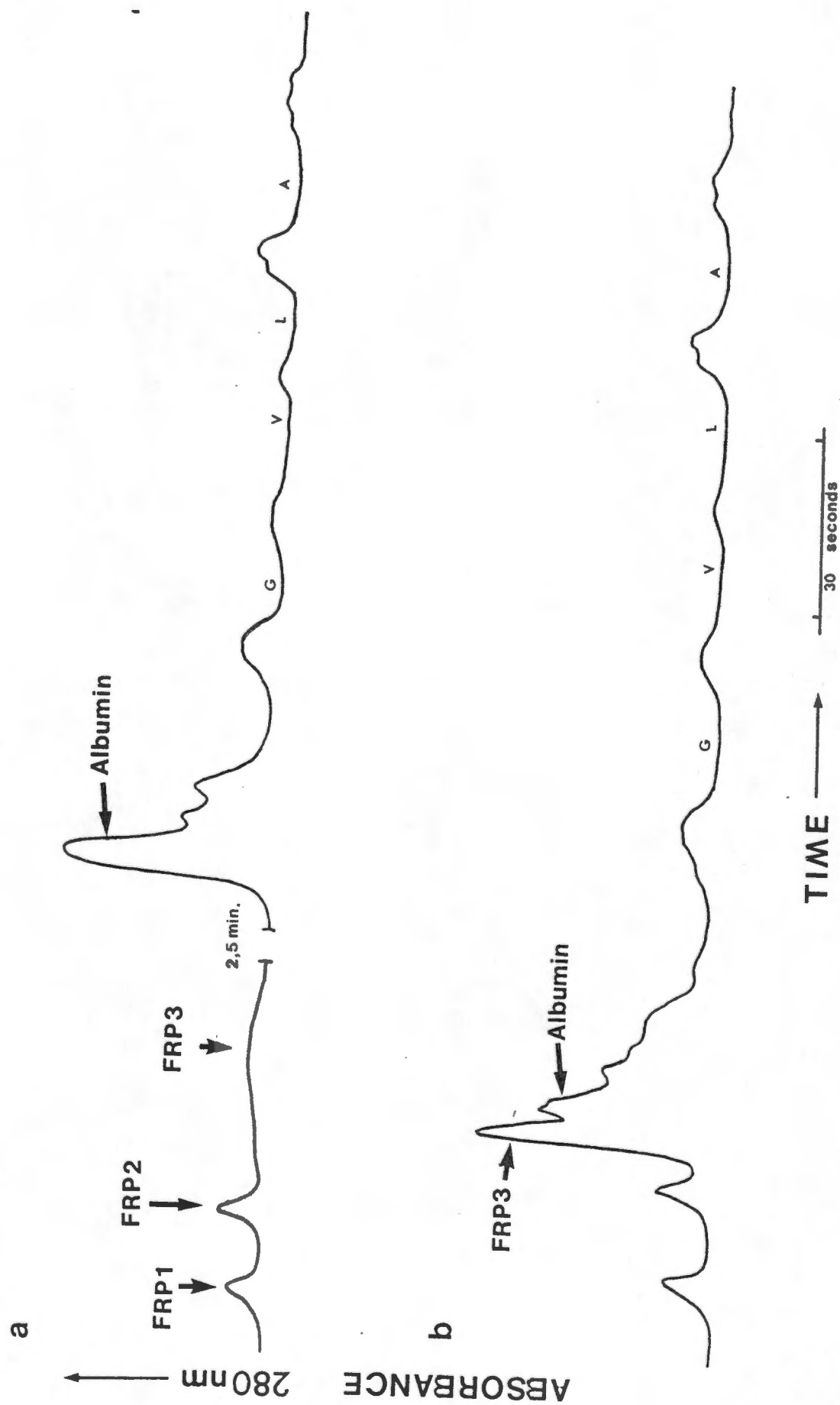

ABSORBANCE 280 nm
a
b
Electrolyte Impurity
Electrolyte Impurities
G
V
L
A
TIME
30 seconds

FIGURE 4.6

Effect of the (a) MES/Ammediol and (b) HCl/Ammediol leading electrolytes on the separation profile of the CSF and especially the FRP.


a
b
ABSORBANCE
280nm
FRP1
FRP2
FRP3
2,5 min.
Albumin
G
V
L
A
TIME
30 seconds

appear to move isotachophoretically in an "in stack" configuration.

The FRP3 in the chloride system is a narrower peak with a great peak height compared to FRP 3 in the MES system. This is due to the concentration effect of isotachophoresis.

4.2.4 Effect of the Chloride ion in the sample on FRP

The isotachophoretic separation of CSF has shown the presence of two main UV-absorbing regions, the FRP region where substances migrate well ahead of albumin and the main protein profile of CSF (Figure 4.6a). It has previously been shown in 4.2.3 that the position of these FRP changes when the MES leading ion is exchanged for a chloride ion. The FRP occupy a position immediately ahead of albumin (Figure 4.6b).

From these results it was concluded that these substances have a mobility greater than the MES ion and less than the chloride ion. Thus, in the MES/Ammediol system it was thought that the FRP must be migrating electrophoretically in an "out of stack" configuration as described by Mikkers and Everaerts (1981).

However, this has led to certain problems as it is known that a 10 µL sample occupies a length of 50 mm in a 0,5 mm

capillary. These FRP should, therefore, also occupy an equivalent length as zone electrophoresis does not allow for concentration. The resultant peaks would be broad and diffuse. However, examination of the FRP has shown that these substances are concentrated into zones less than 1 mm. Thus, isotachophresis must be occurring.

As there is sodium chloride in the sample, the chloride ions present may account for the partial isotachophoretic concentration of the FRP. The highly mobile chloride ions would migrate through the MES leading electrolyte and, in so doing, superimpose their physical properties on the system and take over the role of the leading ion. The fraction of the chosen leading ion, MES, that has been overtaken by the chloride ion then acts as a spacer between the FRP and the main protein region. This hypothesis is examined below.

4.2.4.1 Methods

A 1 mL normal CSF sample was dialyzed for 18 hours at 4°C against distilled water in a dialysis membrane tube with a molecular weight cutoff of 1 000 daltons (Spectrum Medical Industries, Inc., U.S.A.). Ten µL of the dialyzed CSF with spacers was examined in the MES/Ammediol system and re-examined in the same system with the addition of a 1 µL saturated aqueous solution of uric acid and a 1 µL saturated aqueous solution of folic acid which are known to be FRP1 and FRP3 (Chapter VI).

The dialyzed CSF with folic acid and uric acid and 1 µL 5 mM MES was also examined in the chloride leading electrolyte system in order to prove that MES acts as a spacer between the FRP and main protein region.

4.2.4.2 Results

Figure 4.6a shows the isotachopherogram of the undialyzed normal CSF in the MES/Ammediol system with the FRP, two of which have been identified as folic acid (FRP1) and uric acid (FRP3) (See Chapter VI).

The dialyzed CSF sample shows the absence of FRP in the MES/Ammediol system (Figure 4.7a), indicating that their molecular weights are less than 1000 daltons. As two of the FRP are now known, folic acid and uric acid were added and re-examined in the MES/Ammediol system. Figure 4.7b shows the presence of two broad and diffuse UV absorbing zones of folic acid and uric acid and indicating that, in the absence of chloride ion in the CSF, these two substances migrate zone electrophoretically and are not concentrated. The length of each FRP zone is equivalent to 50 mm.

Figure 4.8a shows the position of folic acid and uric acid in relation to the albumin peak in the chloride leading ion system. These two substances occupy a position in contact with the albumin peak. However, when 1

FIGURE 4.7

(a) Isotachopherogram of dialyzed normal CSF in MES/ Ammediol system showing the absence of FRP.

(b) Isotachopherogram of dialyzed normal CSF in MES/ Ammediol system with added folic acid and uric acid, showing that these two acids migrate electrophoretically in an "out of stack" configuration.

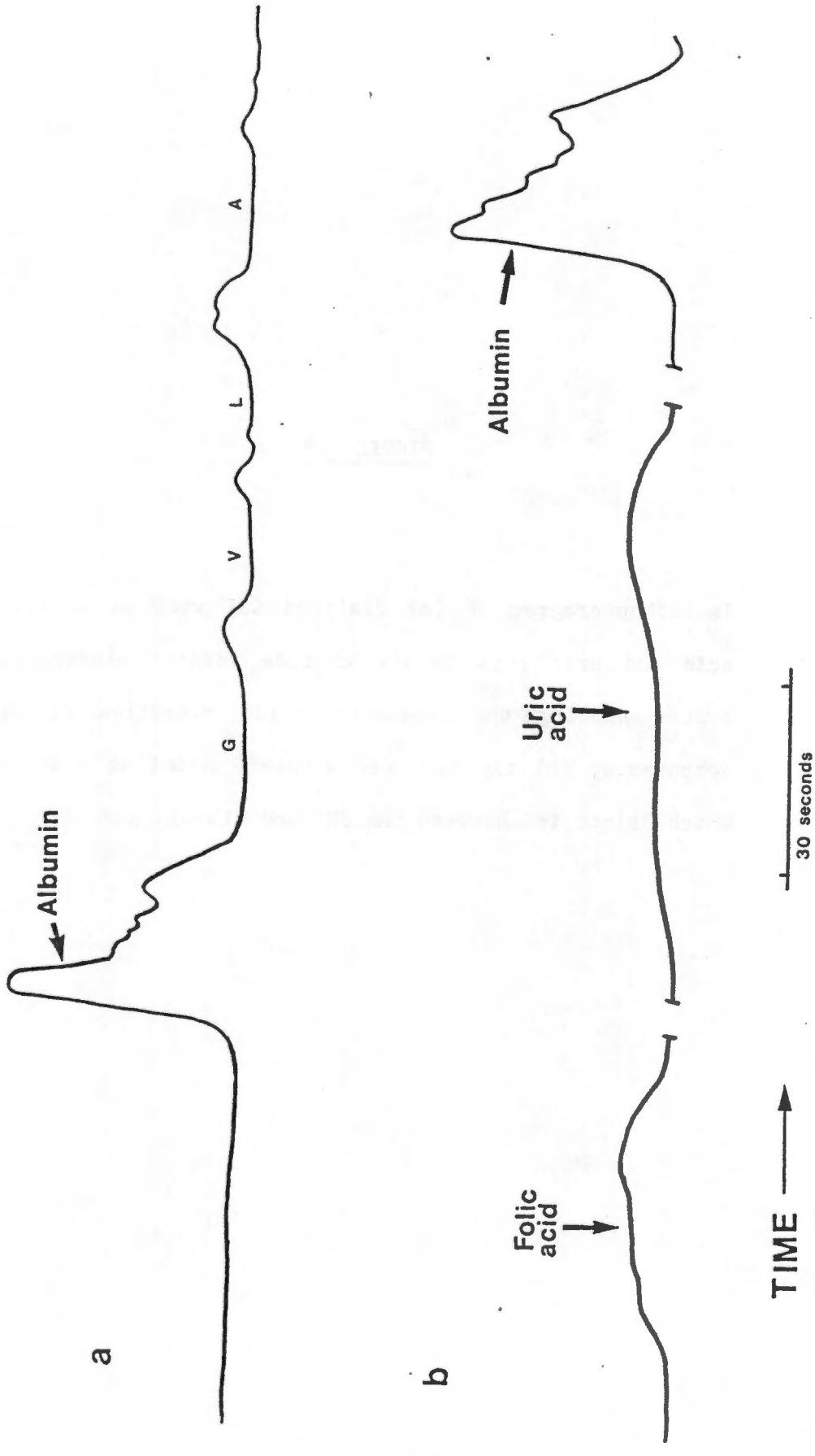
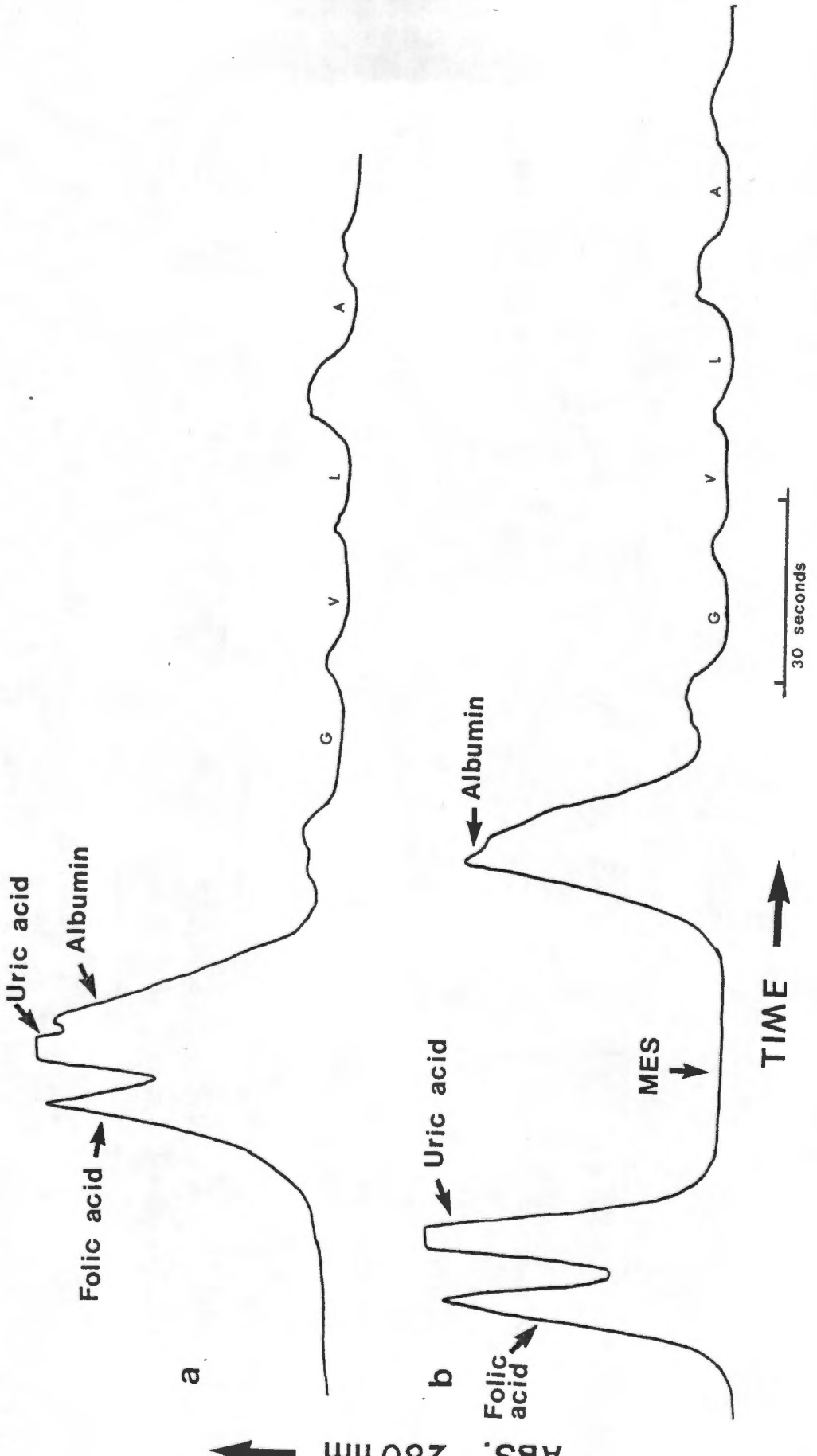

a
Albumin
G
V
L
A
b
Folic acid
Uric acid
Albumin
30 seconds
TIME
ABS. 280 nm

FIGURE 4.8

Isotachopherogram of (a) dialyzed CSF with added folic acid and uric acid in the choride leading electrolyte system showing the isotachophoretic migration of all components; (b) the same CSF with MES added as a spacer which "slots in" between the FRP and albumin peak.


ABS. 280 nm
a
Uric acid
Albumin
Folic acid
G
V
L
A
b
Albumin
Uric acid
MES
Folic acid
G
V
L
A
30 seconds
TIME

µL 5 mM MES was added, this ion acts as a spacer separating the FRP from the albumin peak (Figure 4.8b).

4.2.5 Amino Acid and Ampholyte Spacers

4.2.5.1 Method

10 µL unconcentrated CSF was examined in the anodic MES/Ammediol electrolyte system without spacers. The effect of the addition of 7 µg of the four amino acid spacers, glycine valine, leucine and β alanine to the CSF was examined.

Ampholytes, with different pH ranges, pH 3,5-5, pH 6-8 and pH 9-11, were examined and their effect on the separation pattern noted. The best mixture was determined.

Optimum concentrations of the mobility gradient was established by adding increasing volumes of a 1% mixture of ampholytes to the CSF.

4.2.5.2 Results

Unconcentrated CSF, examined without spacers, show three major peaks. The albumin fraction may be identified with certainty. The last peak is probably the gammaglobulin region (Figure 4.9a).

FIGURE 4.9

Effect of amino acid spacers and different ampholyte pH ranges on the separation profile of the CSF.

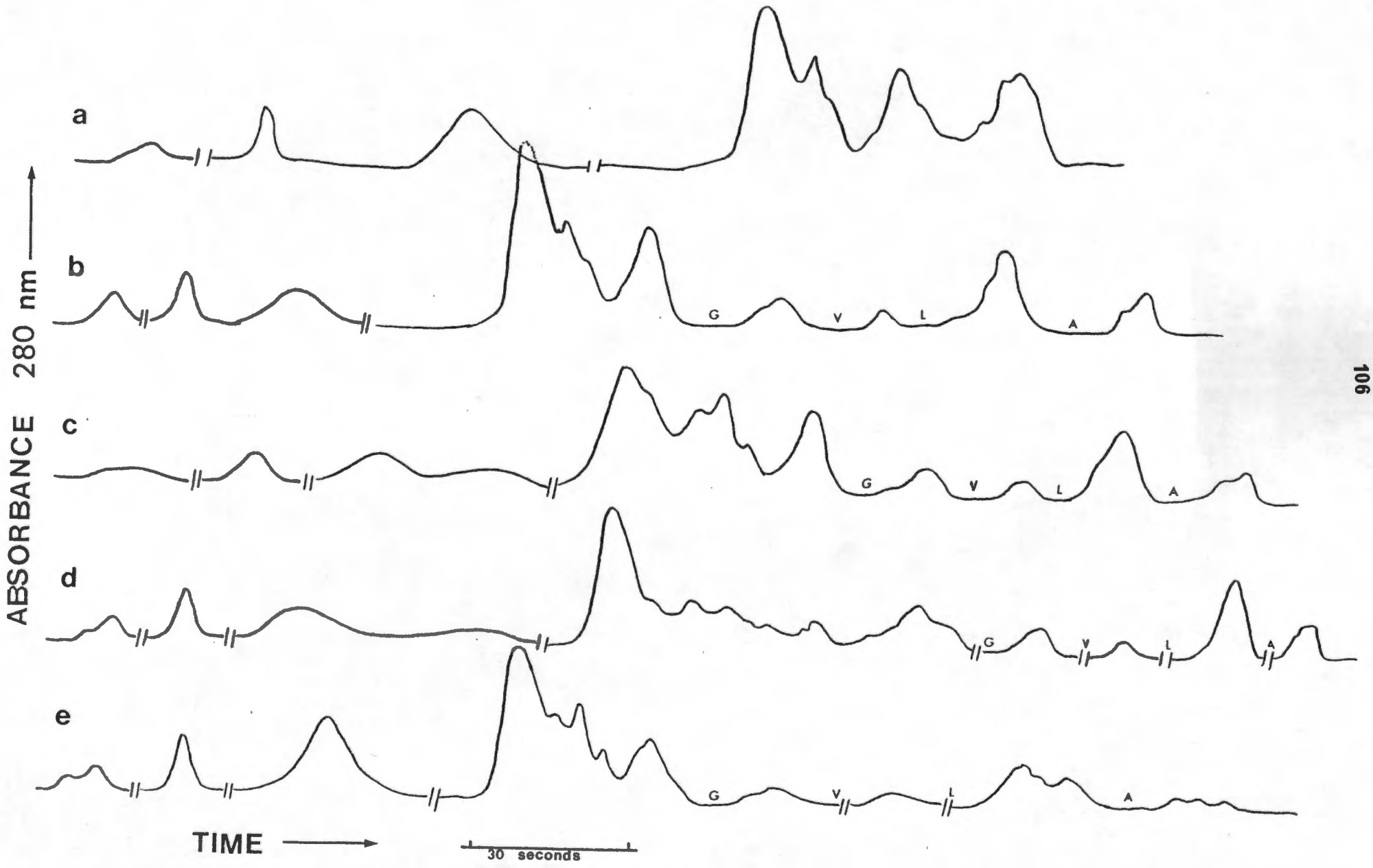
(a) No spacers.

(b) Addition of 4 µL of 7 µg solution of each of the amino acid spacers, glycine, valine, leucine and β alanine.

(c) Addition of 0,6 µL 1% pH 3,5-5,0.

(d) Addition of 0,6 µL 1% pH 6-8.

(e) Addition of 0,6 µL 1% pH 9-11.


a
b
c
d
e
G
V
L
A
ABSORBANCE 280 nm
TIME
30 seconds

The addition of the 4 amino acids divides the CSF proteins into 5 UV-absorbing zones with the immunoglobulin region being split into three zones bounded by valine, leucine and β alanine (Figure 4.9b). The amino acids do not slot into the FRP region.

The addition of 1% ampholytes to the CSF with amino acid spacers provides further information, with certain peaks being spread out, depending on the pH range used. Although only a 2 pH-unit range was used in each case, the separation patterns show clearly the effect of the mobility gradient over a much wider range.

The effect of the addition of low pH range - e.g. pH 3,5-5, is mainly seen in the FRP and albumin region (Figure 4.9c), while that of pH 9-11 is mainly in the immunoglobulin region (Figure 4.9e).

The most appropriate pH for an overall profile was a mixture of equal volumes of 1% pH 6-8, 7-9 and 9-11 (Figure 4.10).

The concentration of the selected mixture is also critical, as the addition of too much ampholytes dilutes the sample zone and information gained by the addition of ampholytes is lost. The effect of increasing the amount of ampholyte on the CSF separation pattern is seen in Figure 4.11.

FIGURE 4.10

Effect of amino acid spacers, glycine (G), valine (V), leucine (L) and β alanine (A) and the ampholyte mixture, 1% pH 6-8, 1% pH 7-9, 1% pH 9-11 mixed together in equal parts.

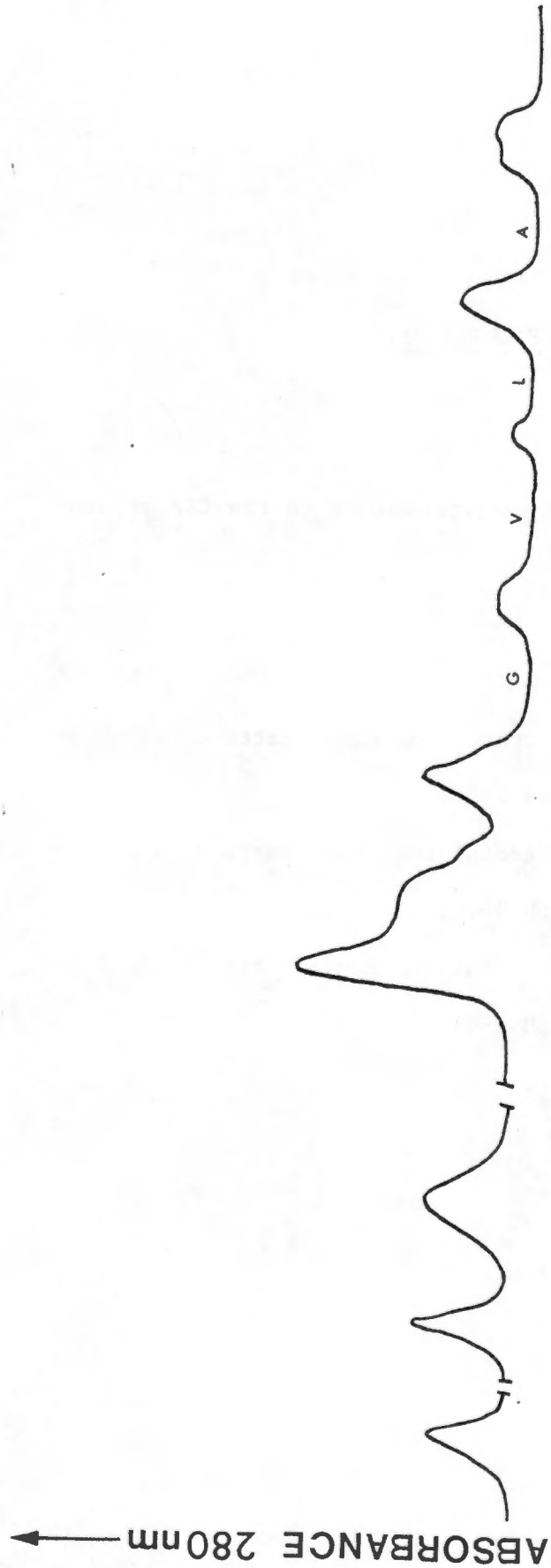

ABSORBANCE 280nm
TIME
30 seconds
G
V
L
A

FIGURE 4.11

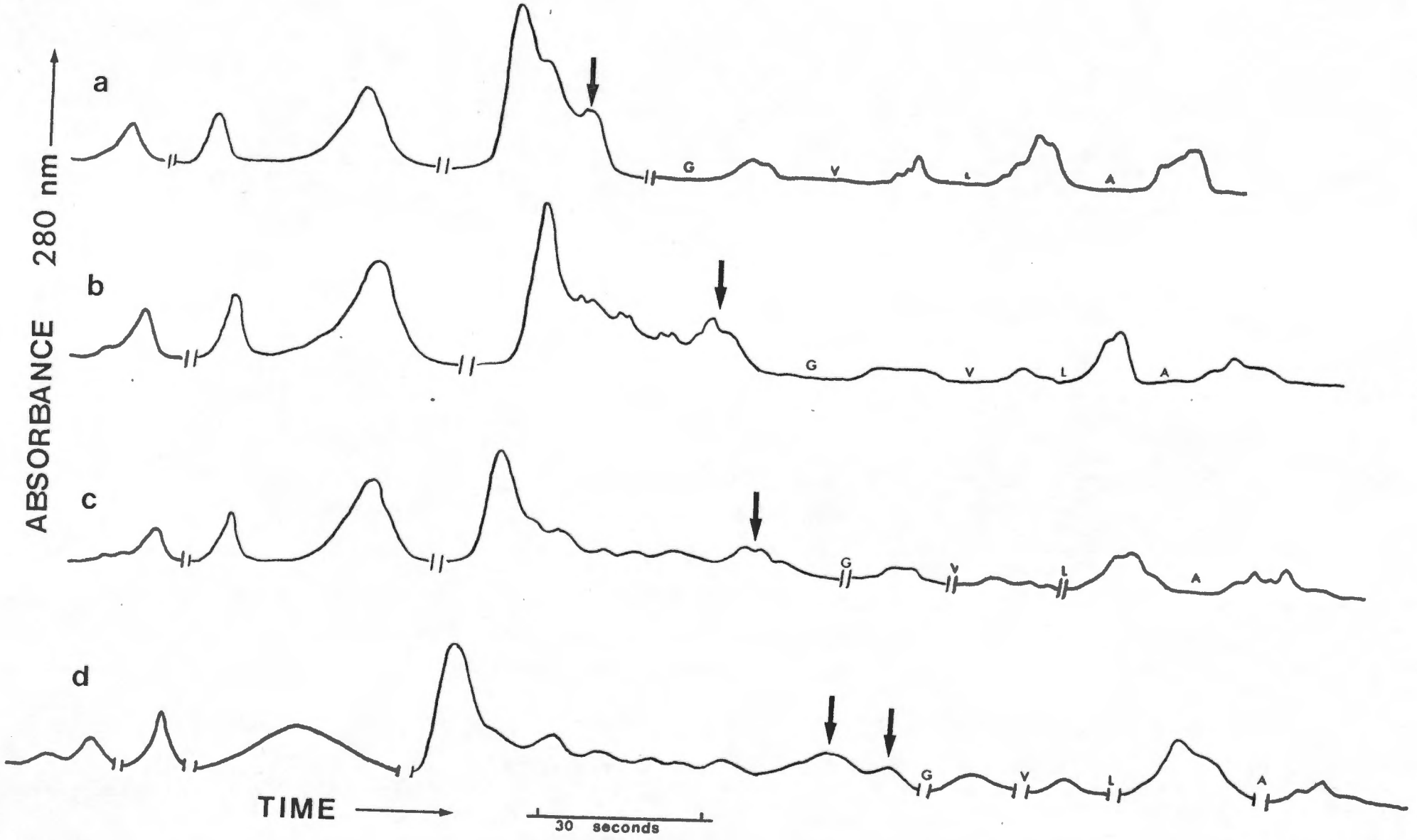
Effect of increasing ampholyte volume on the CSF separation profile.

(a) No ampholytes.

(b) 1 µL of a mixture containing equal parts of 1% pH 6-8, 1% pH 7-9, 1% pH 9-11.

(c) 2 µL of a mixture containing equal parts of 1% pH 6-8, 1% pH 7-9, 1% pH 9-11.

(d) 3 µL of a mixture containing equal parts of 1% pH 6-8, 1% pH 7-9, 1% pH 9-11.


a
b
c
d
ABSORBANCE 280 nm
G
V
L
A
TIME
30 seconds

As the amount of ampholyte is increased, the albumin peak, peaks between leucine and β alanine and the peak ahead of glycine (See arrows Figure 4.11), are spread out. This is due to ampholytes having the same mobility as the separated component. The peak height is reduced due to the "quenching" effect of the non-UV absorbing ampholytes. The sensitivity and resolution is lost with the separated zones becoming more diffuse.

4.3 DISCUSSION

The criterion for isotachophoretic separation is that two charged constituents will separate if their migration rates/ mobilities are different. This separability is largely dependent on the physio-chemical characteristics of the constituents and the time allowed for resolution (Mikkers et al., 1979a). For multi-component samples - e.g. complex protein mixtures, optimization is generally difficult as the constituents have a variety of different properties.

As the leading electrolyte plays an important role in establishing the concentration and velocity of the following separated zones, care must be taken in its choice. In the separation of CSF proteins, the MES leading electrolyte system was chosen in preference to the HCl system, as the mobility of the MES ions is lower and, thus, the resultant sample zones are broader. Resolution and detection of these zones, especially if present in low concentration, is enhanced.

The pH of the leading electrolyte is critical, as it must be chosen so that all constituents of interest will have the same charge sign as that of the leading ion and so move toward the anode. As a result, a relatively high pH is required for the examination of most proteins in an anionic system. For the separation of gammaglobulins, pH values of above 9 are required. Mikkers et al. (1979a), however, show that a low pH of both sample and leading electrolyte may favour resolution in an anionic system as a better mobility ratio is established. This is not possible for proteins.

Mikkers and Everaerts (1981) also point out that working at a high pH in the anionic system, a multiconstituent electrolyte system is created with multiple moving boundaries and isotachophoretic subconfigurations, as a result of contamination of the electroylte system with CO_2, $-HCO_3-$ and CO_3. Reproducibility in this case is supposedly poor.

It is preferable to examine the CSF in an anionic system as the whole protein profile is seen, while cationic isotachophoresis is useful in examining only the immunoglobulin region (Kjellin and Hallander, 1978b, 1979a). However, in this case, there are problems with bubble formation and the need to acidify the sample before injection.

The importance of the role that sample ions may play in the isotachophoretic separation and resolution of a sample should

not be underestimated. In the present study it has been found that the chloride ion, present in untreated CSF at a concentration of 125 mM, takes over the role of the selected leading ion, MES, and isotachophoretically regulates both the high mobility folic acid and uric acid components and lower mobility proteins.

The portion of the MES ion in the leading electrolyte that has been overtaken by the chloride ion acts as a spacer between the FRP and the main protein region.

Although the isotachophoretic regulation of all CSF components by the chloride ion from the sample is not as efficient as a leading electrolyte of chloride ions, it is sufficient to concentrate and maintain the isotachophoretic state.

Increasing the volume of CSF injected into the system resulted in longer analysis times due to the presence of an increased sodium chloride load. Mixed zones are also detected as the capillary length is insufficient for complete separation to occur before the zones reach the detector.

The maximum load capacity of unconcentrated CSF in this system with a 23 cm capillary was found to be 20 µL with 10 µL,

giving satisfactory results. If very small volumes and/or constituent concentrations are analyzed, there is a decrease in the resolution due to the short zone lengths obtained. The reliable detection of such zones is difficult. Mikkers et al. (1979a) have found that zone lengths less than 0,05 mm result in a "zone profile" or spike, rather than a zone length being measured.

These factors depend on the detector and, in practice, the sensitive UV detector is most often used.

As a result of the longer analysis times, there may be a progressive breakdown of the steady state configuration (Mikkers et al., 1979a). However, the time period of 30 - 90 minutes required for the separation of CSF proteins did not produce this effect.

The current plays no role in improving the separation of the components. However, it does influence the analysis time with an increased current reducing the time of separation. Analysis time is inversely proportional to the current applied to the system.

Mikkers et al. (1979a) show that a certain number of coulombs are required to separate a given sample but the time interval in which this amount is delivered is immaterial. There are a number of factors affecting this time interval which include capillary length and sample volume.

Theoretically, the current should be as high as possible but under experimental conditions a compromise must be found. The increased velocity results in a loss of resolution as these shorter zones are difficult to record, being outside the limits of the detector. High currents also result in increased temperatures within the capillary. Convection and diffusion, due to the raised temperature, disturb the zone boundaries and parabolic boundaries also become more evident.

In the present work, the compromise was to start the separation at a high current 200 μA and later reduce it to 50 μA as the sample passes the detector. Thus, analysis time was reduced without adversely affecting the resolution.

Spacers are of great value in the interpretation of the separation profile as there is increased resolution of heterogeneous protein samples (Moberg, 1974; Arlinger, 1975). However, there is a reduction in the efficiency of the separation process (Mikkers et al., 1979a) as the continuous mobility gradient spacers (Ampholytes) separate with the protein zones and dilute and widen these zones which become diffuse (Griffith et

al., 1973). The widening of the protein zone is proportional to the amount of ampholyte added (Arlinger, 1975). However, if too much ampholyte is added, there is a diluting effect and resolution is decreased.

Ideally, the total protein concentration of the CSF should be constant so that a standard amount of ampholyte may be added. At high protein concentrations, a greater amount of ampholyte is required to get the same separation as obtained if a lower protein profile is examined.

It should be noted that the effective mobility of the ions changes with the pH value. Also low pI values of ampholytes creates high charges in the spacing zones and, therefore, higher mobilities (Moberg, 1974).

Wherever possible, only discrete amino acids should be used (Mikkers et al., 1979a) but as the amino acids used as spacers in protein analyses are usually restricted to the physiological amino acids which do not cover the whole range of mobilities (Holloway and Pingoud, 1981), ampholytes have to be used as well. The amino acid spacers also serve as mobility markers and may be used in identifying and defining zones migrating between two spacers.

Using the amino acids, valine and leucine, an unusual elevated

UV-absorbing peak was found in a number of viral meningitis CSF samples examined (See Chapter VII).

The preparation of spacers specifically designed for isotachophoresis of proteins - e.g. synthetic peptides (Holloway and Pingoud, 1981), may improve the resolution significantly (Griffith et al., 1973) as ampholytes are not ideally suited to isotachophoretic separations.

The factors affecting the separation capacity and resolution are many and varied. One must look for optimal conditions which may be a time-consuming process. Unlike isoelectric focusing and discontinuous electrophoresis, the isotachophoretic system is not a "one off" system where good separation with any electrolyte and spacing mixture may be immediately obtained. A knowledge of the basic physico-chemical properties of the electrolytes and sample constituents is essential.

CHAPTER V

QUANTITATIVE STUDIES

5.1 INTRODUCTION

In capillary isotachophoresis, quantitative as well as qualitative studies are possible. At a steady state, the separated zones are homogeneous. The length of each zone is directly proportional to the amount of ions present in the zone. This follows from the Kohlrausch regulating function which shows that, at equilibrium, the concentration of the sample ions is directly proportional to the leading ion concentration. Thus, the ionic concentration in each zone is constant for a given electrolyte system. Should the ionic concentration of a sample zone be greater than the leading ion concentration, it will be automatically diluted, with a consequent increase in the zone length.

Quantitative studies may be performed using any of the detectors available. Svoboda and Vacik (1976) showed that as the UV detector is so specific, it may be used to accurately detect concentrations ten times lower than the conductivity detector. The zone length, as measured by the UV detector, is linearly related to the concentration only if it is longer than the slit width (Svoboda and Vacik, 1976; Ryšlavý et al., 1978a).

It is possible to quantitate small amounts of components with zone lengths that do not fill the slit width completely, by measuring the peak height (Arlinger, 1974; Svoboda and Vacik, 1976). This parameter reflects the molar absorbance (a product of the absorptivity and the molecular weight of a substance) at a given concentration. It is, however, necessary that the zones adjacent to that of interest are long enough to fill the UV slit width. If not, quantitation of the zone becomes difficult. Where small peaks and peaks near the maximum height are obtained, reproducibility is poor (Hjalmarsson and Baldesten, 1981).

The integration of the total area under the curve is also linearly related to the concentration and has been used by Gower and Woledge (1977), Delmotte (1977) and Svoboda et al. (1983) for quantitation.

Where low concentrations of substances are present in a complex solution, it is possible to increase the detection and thus permit quantitation by either selecting detectors of increased sensitivity and/or one that is more specific. The sample volume may also be increased. However, to obtain complete separation with an increased load, the capillary length must be increased or counterflow of the leading electrolyte must be used. Both these methods have disadvantages. With the former a higher potential is required with resultant adverse heating effects while, with the latter, analysis times

are increased, and electrolytic products may interfere with the interpretation of the final result.

Ryšlavý et al. (1978a) used a "continuous sampling method" to increase the total volume of the sample so that initially dilute sample components may be detected and measured. This quantitative technique increased the operational capability of isotachophoresis towards the lower concentrations by at least one to two orders of magnitude.

Everaerts et al. (1979) and Verheggen et al. (1979) overcame the problem by using a column coupling device which consists of a specially-constructed large bore pre-separation tube coupled to the conventional narrow bore tube. This enabled high sample loads to be applied, without substantially affecting the analysis time; high ratios of concentration between sample species are permitted and different electrolyte systems could be applied in the pre-separation and separation compartments.

Beckers and Everaerts (1972a) and Everaerts et al. (1973) described a calibration constant, Kcal, which obviated the necessity of constructing calibration curves for each ionic species under investigaton. The constant is defined as :

$$K_{cal} = \frac{V_i \times C}{C^* \times L^*}$$

where Vi is the volume of the sample injected (mL); C, the concentration of a particular ionic species (mole/cm^3), C* the actual concentration of the ionic species in its zone (mole/cm^3) and L* the zone length of the particular ionic species, measured in seconds.

Everaerts (1974) measured the zone lengths of nitrate, chlorate and acetate at various leading electrolyte concentrations and determined calibration constants for each. Deviations from the average calibration constant were ascribed to variations in the introduction of the sample. The highest linearity and reproducibility was found when small amounts of sample were introduced, resulting in short zone lengths.

Limitations of this calibration constant method are that identical working conditions are required - e.g. current density, counterflow, temperature and composition of leading electrolyte. The absolute concentration of all ionic species in the leading electrolyte, ionic mobilities, dissociation constants and absolute amount of sample used, must be known (Boček et al., 1974).

Boček et al. (1974) used correction factors to simplify relationships between the amount of ionic species in a zone and the corresponding zone length in such a way that it may be possible to convert any zone length recorded into abitary mole units by using a factor dependent only upon the respective ionic species and independent of the working conditions. The ratio of the relative correction factor DRX, (defined as the molar dilution of species x relative to that of a suitable reference species, R, separated simultaneously) and the zone length (Lx) of species x, was found to be directly proportional to the molar amounts of the respective species and represent directly the composition of the sample in arbitary mole units.

In this Chapter, the relationships between peak width, peak height and peak area and the concentration of human albumin and IgG are shown. These standard curves are compared with those obtained from area measurements of albumin and IgG peaks in the CSF pattern. This was to determine if the isotachopherogram of CSF could be used to quantitate proteins - e.g. albumin and IgG. The relationship between increasing sample volume and peak area is also investigated. The reproducibility of the technique is demonstrated.

5.2 METHODS AND RESULTS

5.2.1 Human Albumin Standard Curves

5.2.1.1 Method

The concentration of a human albumin (Sigma Chemical Co., U.S.A.) solution in normal saline, was determined by the extinction coefficient method at 280 nm ($E^{1\%}_{1cm\ 280nm}$). This is claimed to be an accurate method for determining concentration and a 1% (m/v) human albumin solution has an absorbance at 280 nm of 6,67 for a 10 mm path length (Little and Donahue, 1968).

The peak width (mm), peak height (mm) and peak area (arbitrary integrated units) of dilutions of a human albumin solution with spacers, were determined by injecting 10 µL into the capillary into the leading electrolyte using a Hamilton syringe. The isotachophoretic analysis was recorded. The electrolyte solutions used were those described in Chapter III.

5.2.1.2 Results

The isotachopherograms (Figure 5.1) of some of the dilutions of human albumin show the reduction in peak width, height and area as the concentration is reduced. Although three times crystallized, essentially globulin-free human albumin was used, contaminating components are present.

FIGURE 5.1

Isotachopherograms of 10 μL of human albumin at various dilutions.

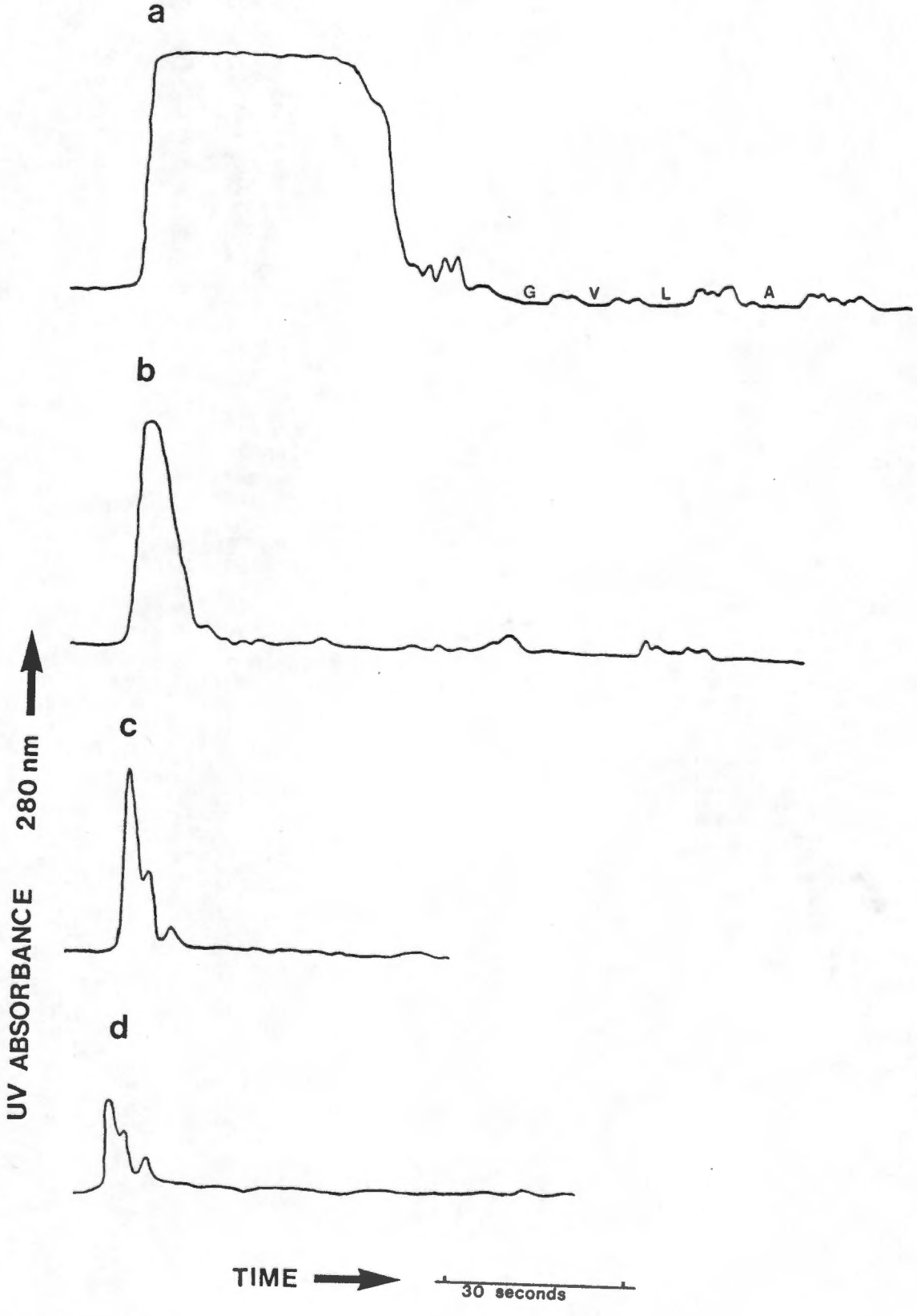
(a) 474 mg/dL

(b) 59,0 mg/dL

(c) 20 mg/dL

(e) 9 mg/dL

(G - glycine, V - valine, L - leucine, A - β alanine)


a
G
V
L
A
b
c
d
UV ABSORBANCE 280 nm
TIME
30 seconds

Each dilution was examined in duplicate and the peak width, peak height and peak area recorded (Table 5.1). At high albumin concentrations, the area could not be determined as it was above the sensitivity set for the integrator. A good correlation exists between the concentration and the various parameters - i.e. peak width, peak height and area.

The computed "best fit" (least squares method) line (Table 5.2) for each of the parameters was used to graphically illustrate these relationships (Figure 5.2 and 5.3). Where the straight line relationship between concentration and peak width deviates, the peak height may be used as a measure of concentration. A direct proportionality also exists between peak area and concentration.

5.2.2 Human IgG Standard Curve

5.2.2.1 Method

The concentration of a human IgG (Sigma Chemical Co., U.S.A.) solution in normal saline was determined in a spectrophotometer, using the extinction coefficient method ($E^{1\%}_{1cm\ 280nm}$). The E 1%/280 of a 1% (m/v) human IgG solution is 14,3 $\pm$ 0,2 for a 10 mm path length (Little and Donahue, 1968)

10 µL of dilutions of human IgG with spacers were examined by isotachophoresis and the areas of the three differ-

TABLE 5.1 - DUPLICATE RESULTS OF PEAK WIDTH, PEAK HEIGHT AND PEAK AREA OF DILUTIONS OF HUMAN ALBUMIN

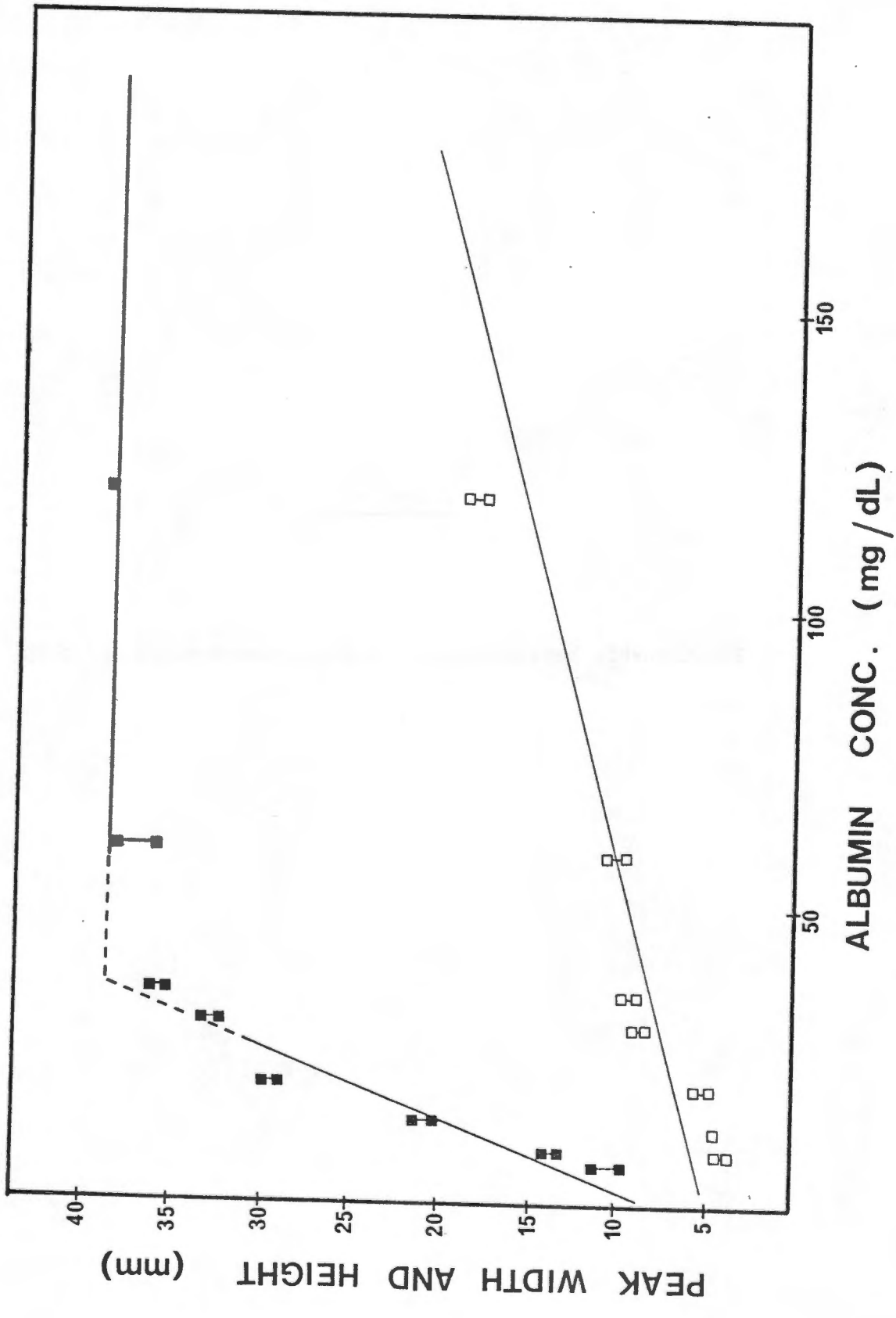
ALBUMIN CONC. (mg/dL)	PEAK WIDTH (mm)	PEAK HEIGHT (mm)	PEAK AREA (Intg. units)
474	44,0	38,0	-
236	32,0	39,0	-
	30,0	39,5	-
118	19,5	39,0	-
	17,5	39,0	-
59	10,5	38,0	182,2
	11,5	36,5	188,2
35	9,0	36,0	113,8
	10,0	36,5	117,8
30	8,5	33,5	90,2
	9,0	32,5	92,7
20	6,5	29,5	74,2
	5,0	30,0	73,7
12,8	4,5	21,5	44,1
	4,5	20,0	41,2
9,0	4,5	13,5	34,3
	4,0	14,0	35,1
7,0	4,5	9,5	27,2
	4,0	11,0	32,1
Correlation Coefficient	0,9731	0,9561	0,9967
Y Intercept	4,9131	7,5889	7,725
Slope	0,0932	0,8740	3,005

TABLE 5.2 - COMPUTED "BEST FIT" RESULTS OF PEAK WIDTH, PEAK HEIGHT AND PEAK AREA FOR VARIOUS ALBUMIN CONCENTRATIONS

ALBUMIN CONC. (mg/dL)	PEAK WIDTH (mm)	PEAK HEIGHT (mm)	PEAK AREA (Intg. units)
300	32,8	-	-
250	28,2	-	-
200	23,5	-	-
150	18,9	-	-
100	14,2	-	308,3
50	9,6	38,8	157,9
25	7,2	29,4	82,8
20	-	25,0	67,8
15	-	20,6	52,8
10	-	16,3	37,7
5	-	11,9	22,7
2,5	-	9,7	15,2

FIGURE 5.2

Relationship between human albumin concentration and peak width (□——□) and peak height (■——■).


PEAK WIDTH AND HEIGHT (mm)
40
35
30
25
20
15
10
5
50
100
150
ALBUMIN CONC. (mg/dL)

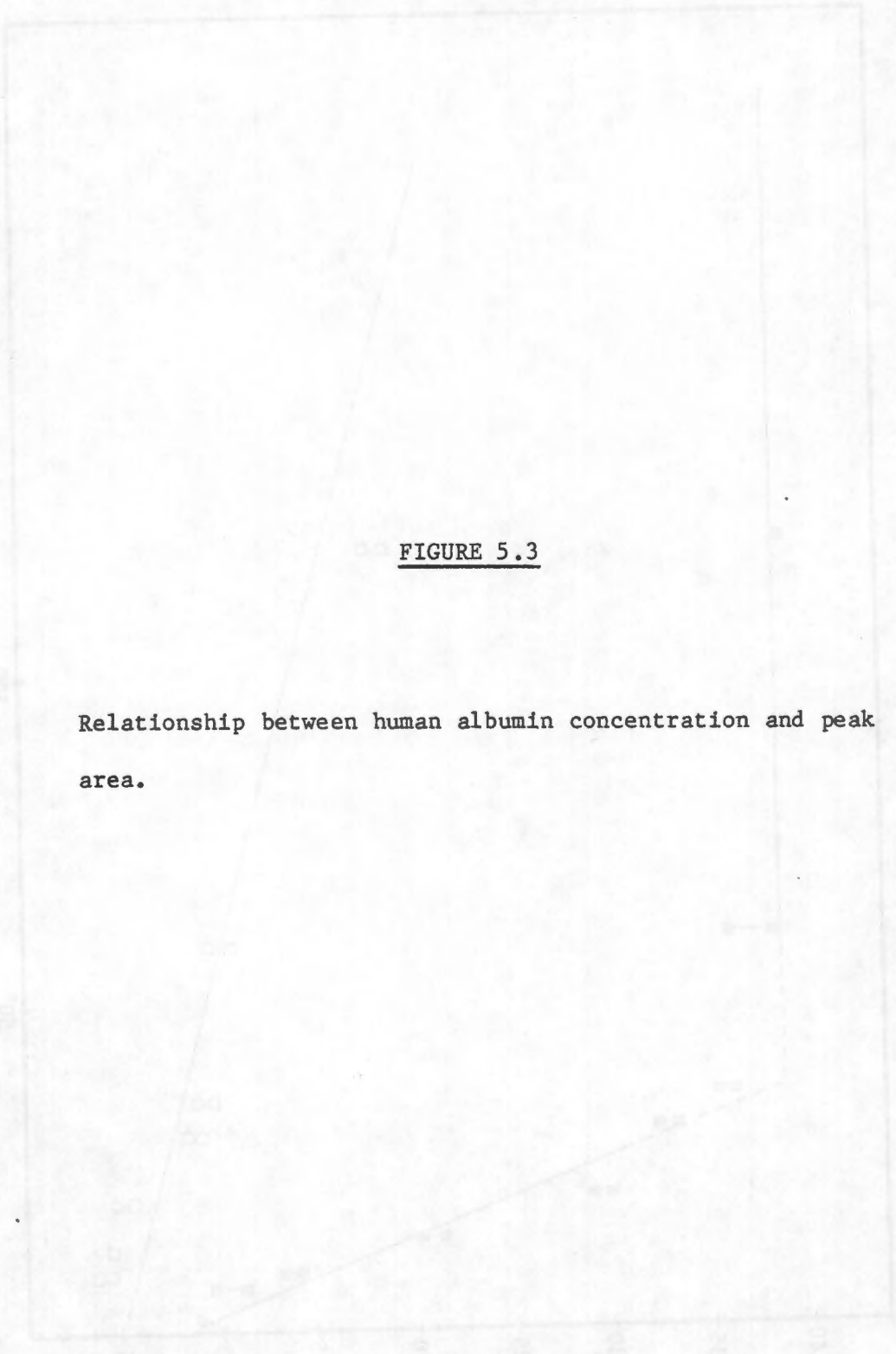
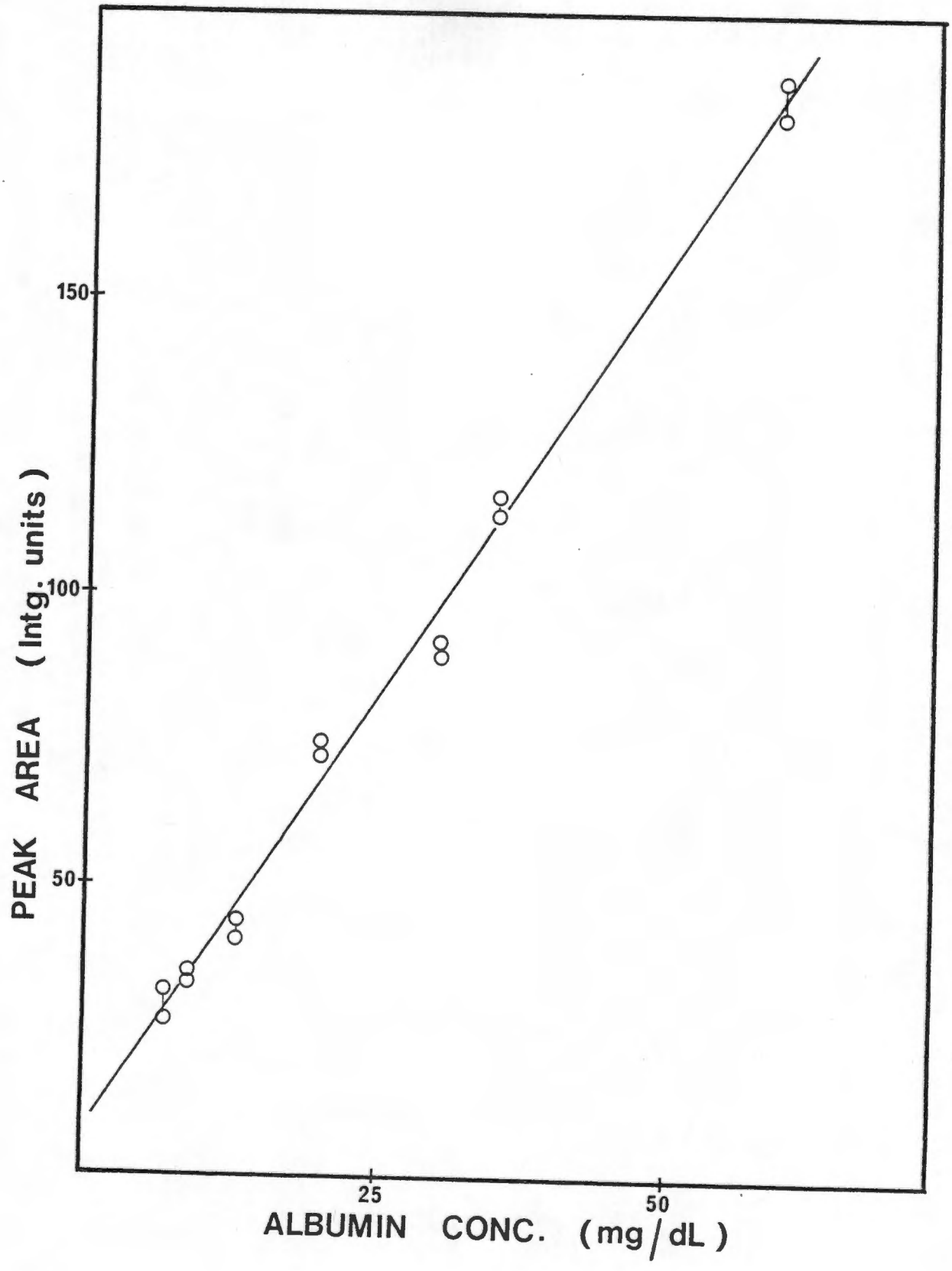


FIGURE 5.3

Relationship between human albumin concentration and peak area.


150
100
50
PEAK AREA (Intg. units)
25
50
ALBUMIN CONC. (mg/dL)

rent regions bounded by the amino acid spacers valine, leucine and β alanine were recorded, as was the total area.

5.2.2.2 Results

The isotachopherograms of some of the dilutions of IgG show the three different immunoglobulin regions (Figure 5.4). There are contaminating peaks of mobility greater than valine.

The peak areas for the different regions were recorded and a good correlation was found between peak area and concentration (Table 5.3).

Figure 5.5 graphically illustrates these results with the computed "best fit" line (from Table 5.4) being drawn between the points.

5.2.3 Relationship between the Concentrations of CSF Albumin and IgG as determined by Radial Immunodiffusion and Peak Area

5.2.3.1 Method

The IgG and albumin concentrations of a number of normal and multiple sclerosis CSF samples were tested by radial immunodiffusion (RID) (ICL Scientific, Calif., U.S.A.) (See Appendix B). The CSF samples with spacers (as described previously, Chapter III) were also examined by

FIGURE 5.4

Isotachopherograms of 10 μL of human IgG at various dilutions.

(a) 45,1 mg/dL

(b) 33,8 mg/dL

(c) 16,9 mg/dL

(d) 5,6 mg/dL

(G - glycine, V - valine, L - Leucine, A - β alanine)

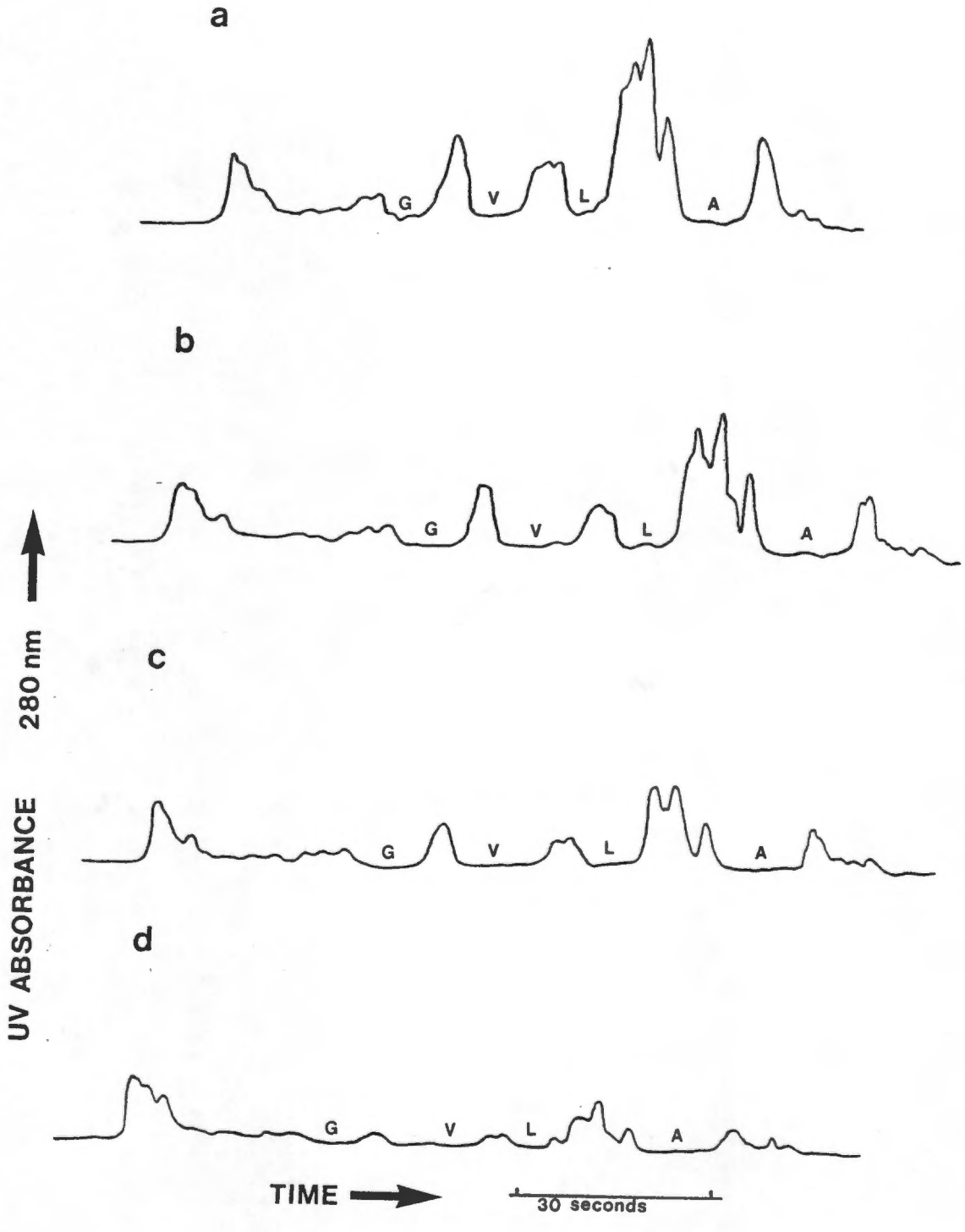

a
G
V
L
A
b
G
V
L
A
c
G
V
L
A
d
G
V
L
A
280 nm
UV ABSORBANCE
TIME
30 seconds

TABLE 5.3 - DUPLICATE RESULTS OF PEAK AREA AND DILUTIONS OF HUMAN IMMUNOGLOBULIN G

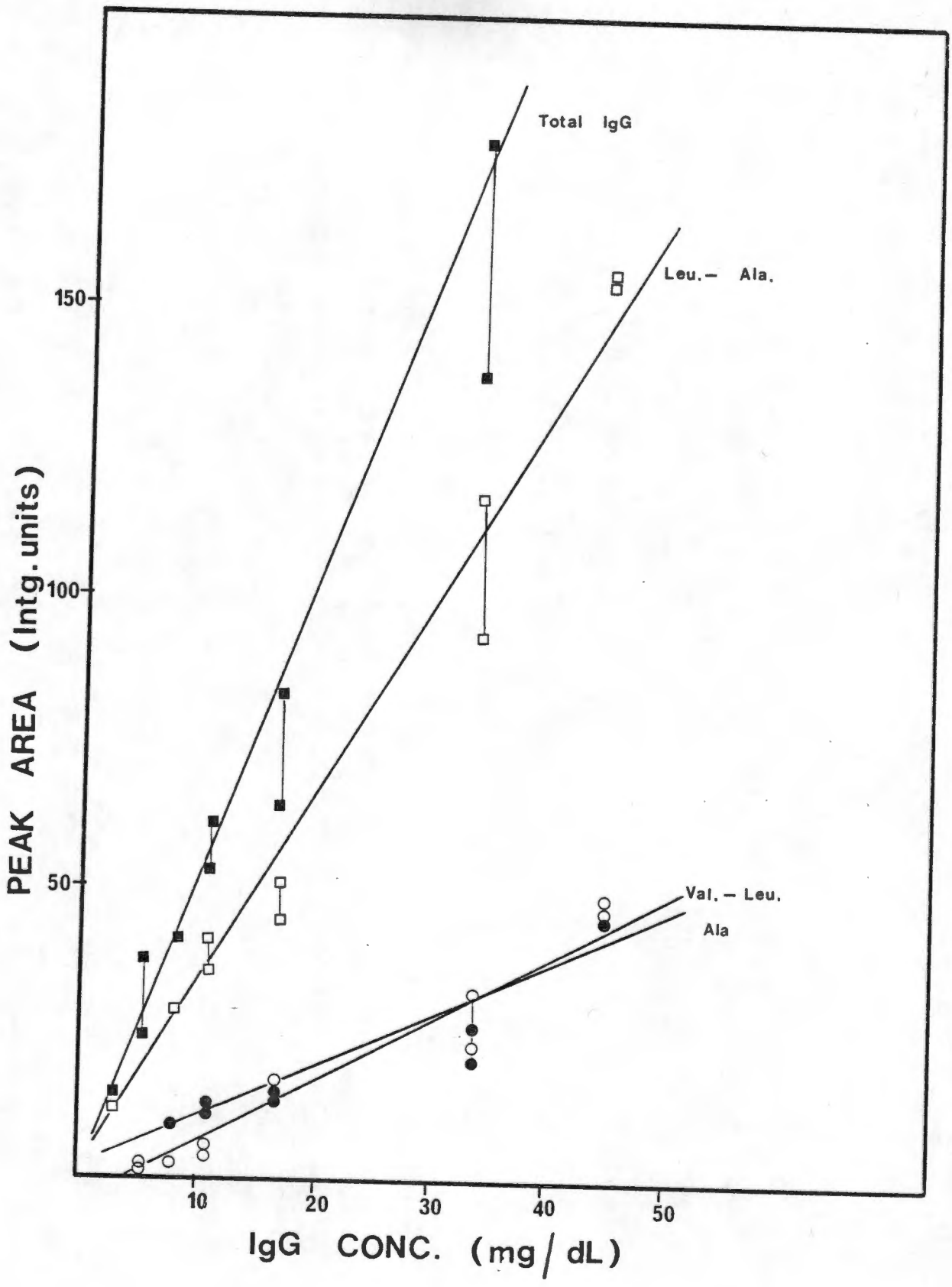
IgG CONC. (mg/dL)	PEAK AREA (Intg. units)			
	VAL - LEU	LEU - ALA	ALA	TOTAL
45,1	46,3	154,2	48,9	249,5
	45,0	155,6	46,4	247,1
33,8	23,8	93,5	21,3	138,8
	32,4	117,3	27,7	177,5
16,9	17,3	51,2	15,3	83,9
	-	45,6	14,0	64,7
11,2	6,1	41,4	13,3	60,8
	4,6	36,0	12,4	53,1
8,4	3,2	29,4	9,8	42,4
	3,3	29,2	9,6	42,2
5,6	1,2	-	-	
	2,7	25,3	9,4	37,4
2,8	1,3	14,2	6,7	22,3
	4,2	12,3	6,6	23,2
Correlation Coefficient	0,9833	0,9892	0,9503	0,9837
Y Intercept	- 4,63	1,2	1,8	- 0,7
Slope	10,6	32,6	8,8	51,81

TABLE 5.4 - COMPUTED "BEST FIT" RESULTS OF PEAK AREA FOR VARIOUS DILUTIONS OF HUMAN IgG REGIONS

IgG CONC. (mg/dL)	PEAK AREA (Intg. units)			
	VAL - LEU	LEU - ALA	ALA	TOTAL
50	48,7	164,4	46,2	258,2
25	22,0	82,8	24,0	128,7
20	16,7	66,5	19,6	102,8
15	11,3	50,1	15,1	76,9
10	6,0	33,8	10,7	51,0
5	0,6	17,5	6,3	25,1
2,5	- 1,9	9,3	4,1	12,1

FIGURE 5.5

Relationship between the concentration of the subfractions of human IgG and peak area. (■——■) total IgG; (●——●) subfraction between the amino acid spacers valine and leucine; (□——□) subfraction between the amino acid spacers leucine and β alanine; (○——○) subfraction of mobility less than the amino acid spacer β alanine.


Total IgG
Leu.– Ala.
Val. – Leu.
Ala
150
100
50
PEAK AREA (Intg. units)
10
20
30
40
50
IgG CONC. (mg/dL)

isotachophoresis and the total IgG and albumin areas were correlated with IgG and albumin concentrations as determined by RID.

5.2.3.2 Results

The isotachopherogram (Figure 5.6) shows a typical normal CSF and multiple sclerosis CSF separation pattern. The areas of the albumin and IgG peaks (indicated by arrows) are recorded in Table 5.5 and Table 5.7, respectively. The correlations are lower than those obtained for the standard curves, 0,888 (albumin) and 0,9058 (IgG). This is due, in part, to the method of determining the albumin and IgG concentration, as the RID method is inherently an imprecise method as inaccuracies in zone diameter measurements occur. These inaccuracies are stated to be ± 20% (Perry et al., 1974).

Figure 5.7 and 5.8 graphically illustrates these results with the computed "best fit" line (Table 5.6 and 5.8) being drawn through the points.

5.2.4 Comparison of the Albumin and IgG Standard Curve Graphs with the CSF Albumin and IgG Graphs

A comparison of the best fit plots for albumin and IgG as determined by the extinction coefficient method and the radial immunodiffusion method (Figure 5.9) shows no relationship between the two sets of graphs.

FIGURE 5.6

Isotachopherograms of (a) typical normal and (b) multiple sclerosis CSF protein profiles.

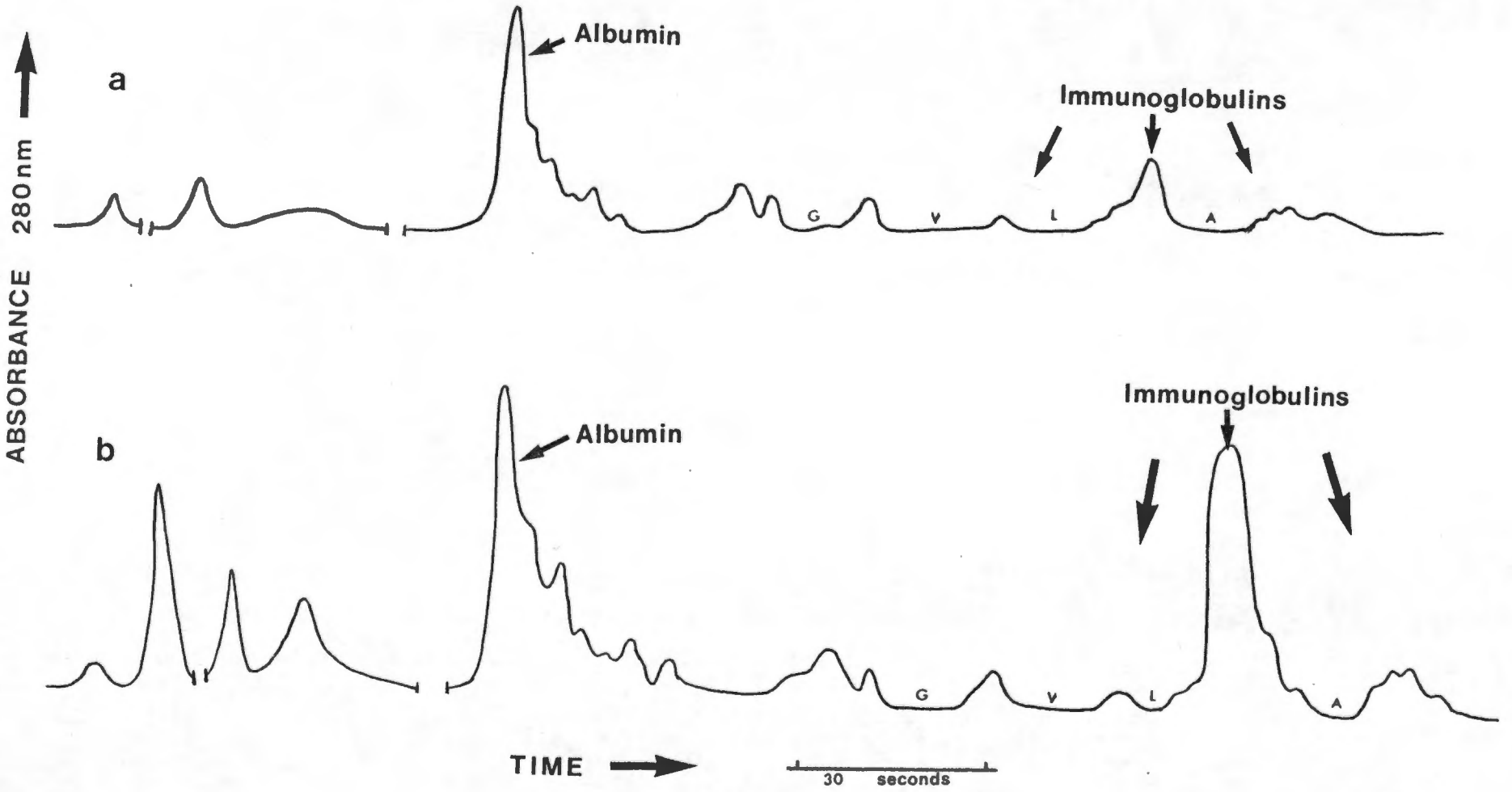

ABSORBANCE 280nm
a
Albumin
Immunoglobulins
G
V
L
A
b
Albumin
Immunoglobulins
G
V
L
A
TIME
30 seconds

TABLE 5.5 - RESULTS OF ALBUMIN CONCENTRATION (mg/dL) AS DETERMINED BY RADIAL IMMUNODIFFUSION AND PEAK AREA OF NORMAL AND MULTIPLE SCLEROSIS CSF SAMPLES

CSF SAMPLES	RID (mg/dL)	PEAK AREA (Intg. units)
NORMAL		
CSF 731	15,5	43,1
CSF 639	16,0	41,3
CSF 867	23,0	54,6
CSF 897	12,0	32,8
CSF 929	26,0	49,1
CSF 854	16,0	40,4
CSF 808	15,0	39,2
CSF 933	19,0	39,3
CSF 943	12,0	30,4
MULTIPLE SCLEROSIS		
V78/927	22,0	53,2
V78/1117	16,0	31,0
V79/3017	28,0	64,4
V78/2217	25,0	50,1
V79/1021	23,0	55,8
V77/1220	17,5	47,8
V77/1766	17,0	33,0
V77/3262	28,0	60,9
V78/1716	19,0	45,8
CSF 906	19,0	43,2
Correlation Coefficient	0,8888	
Y Intercept	10,6	
Slope	17,7	

TABLE 5.6 - COMPUTED "BEST FIT" RESULTS OF ALBUMIN PEAK AREA FOR VARIOUS CSF ALBUMIN CONCENTRATIONS AS DETERMINED BY RADIAL IMMUNODIFFUSION

CSF ALBUMIN (mg/dL)	PEAK AREA (Intg. units)
30	63,7
25	54,9
20	46,0
15	37,1
10	28,3
5	19,4

TABLE 5.7 - RESULTS OF IgG CONCENTRATION (mg/dL) AS DETERMINED BY RADIAL IMMUNODIFFUSION AND PEAK AREA OF NORMAL, AND MULTIPLE SCLEROSIS CSF SAMPLES

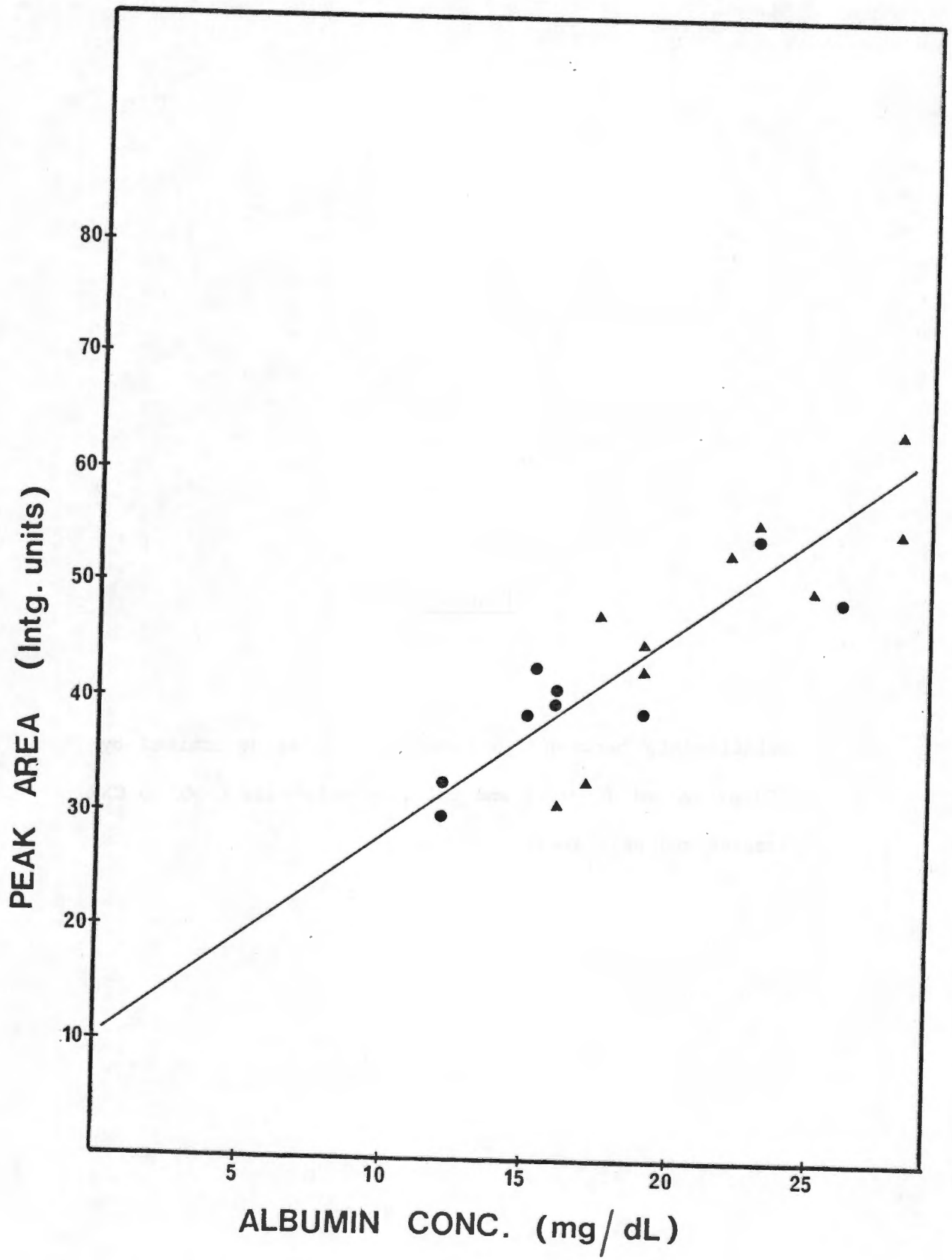
CSF SAMPLES	RID (mg/dL)	PEAK AREA (Intg. units)
NORMAL		
CSF 639	0,8	24,6
CSF 891	1,2	33,3
CSF 867	0,8	36,3
CSF 799	3,8	24,9
CSF 929	2,6	32,3
CSF 944	0,8	20,2
CSF 854	0,8	27,3
CSF 933	2,0	20,0
CSF 884	0,8	34,5
CSF 623	0,8	22,5
MULTIPLE SCLEROSIS		
V76/2878	1,2	25,0
V79/3017	15,0	79,7
V77/1413	7,2	41,1
V78/934	9,6	66,8
V78/1889	7,8	33,7
V78/1920	6,6	48,2
V78/975	6,6	46,2
V77/3366	4,4	44,8
V78/2217	5,6	43,6
V76/1991	10,2	67,7
V77/3262	2,5	27,9
V77/3341	10,5	68,8
V78/1716	2,3	34,1
Correlation Coefficient	0,9058	
Y Intercept	21,9	
Slope	38,2	

TABLE 5.8 - COMPUTED "BEST FIT" RESULTS OF PEAK AREA FOR VARIOUS CSF IgG CONCENTRATIONS AS DETERMINED BY RADIAL IMMUNODIFFUSION

CSF IgG CONC. (mg/dL)	PEAK AREA (Intg. units)
10	60,2
7,5	50,6
5	41,1
2,5	31,5
1,0	25,8
0,5	23,9

FIGURE 5.7

Relationship between albumin concentration as determined by RID of normal (●) and multiple sclerosis (▲) CSF samples and peak area.


PEAK AREA (Intg. units)
80
70
60
50
40
30
20
10
5
10
15
20
25
ALBUMIN CONC. (mg/dL)

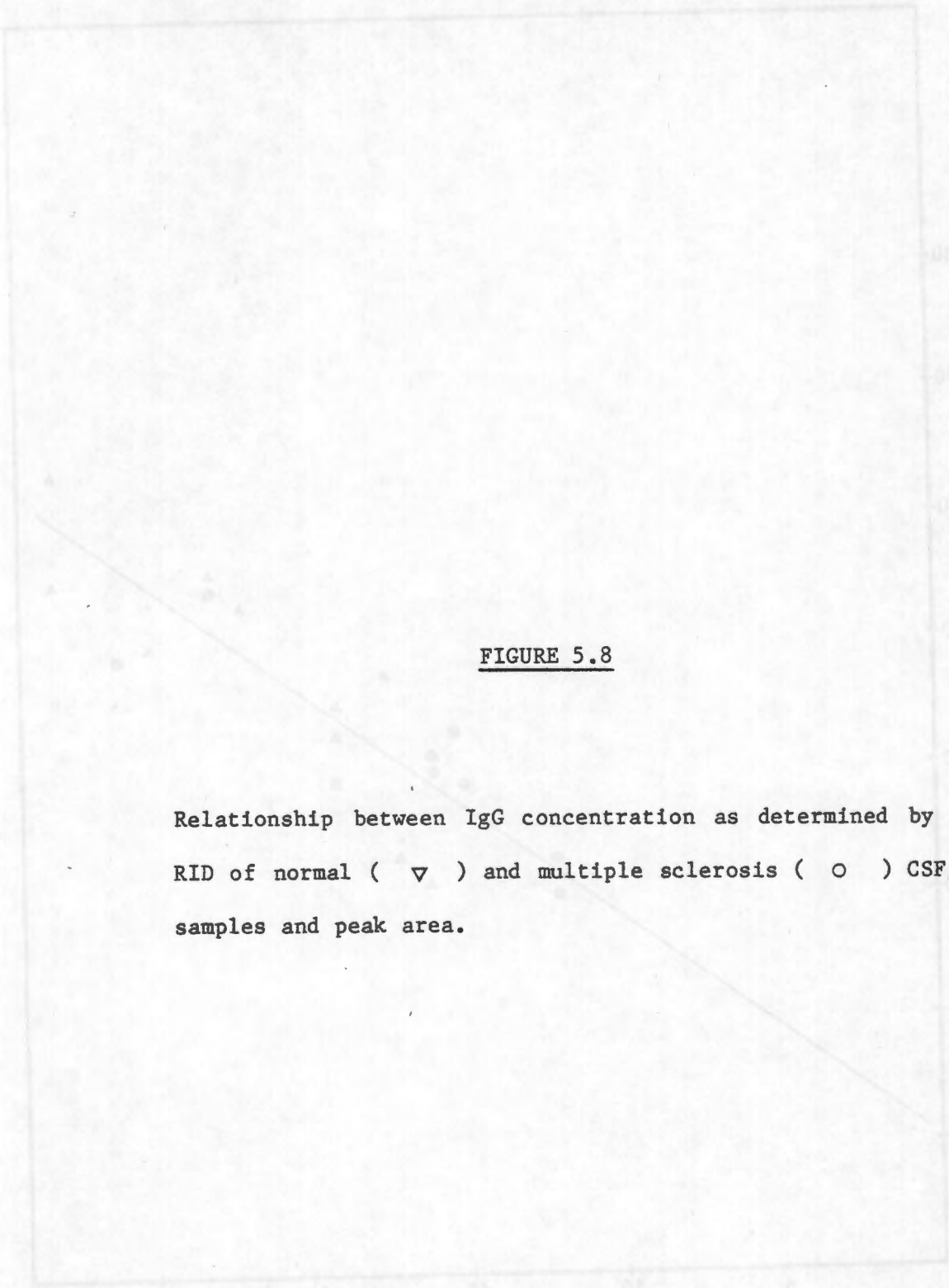


FIGURE 5.8

Relationship between IgG concentration as determined by RID of normal (▽) and multiple sclerosis (○) CSF samples and peak area.

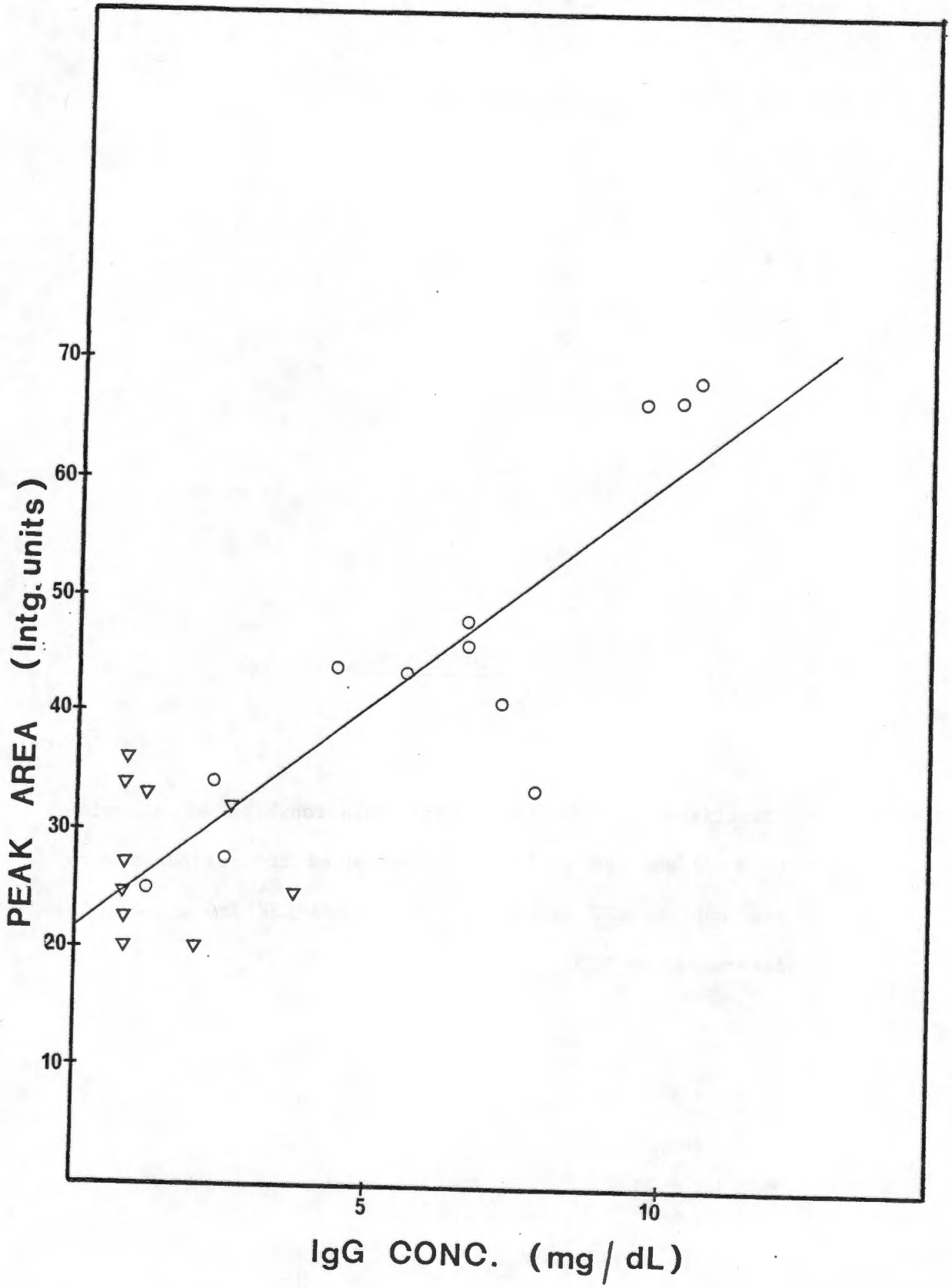
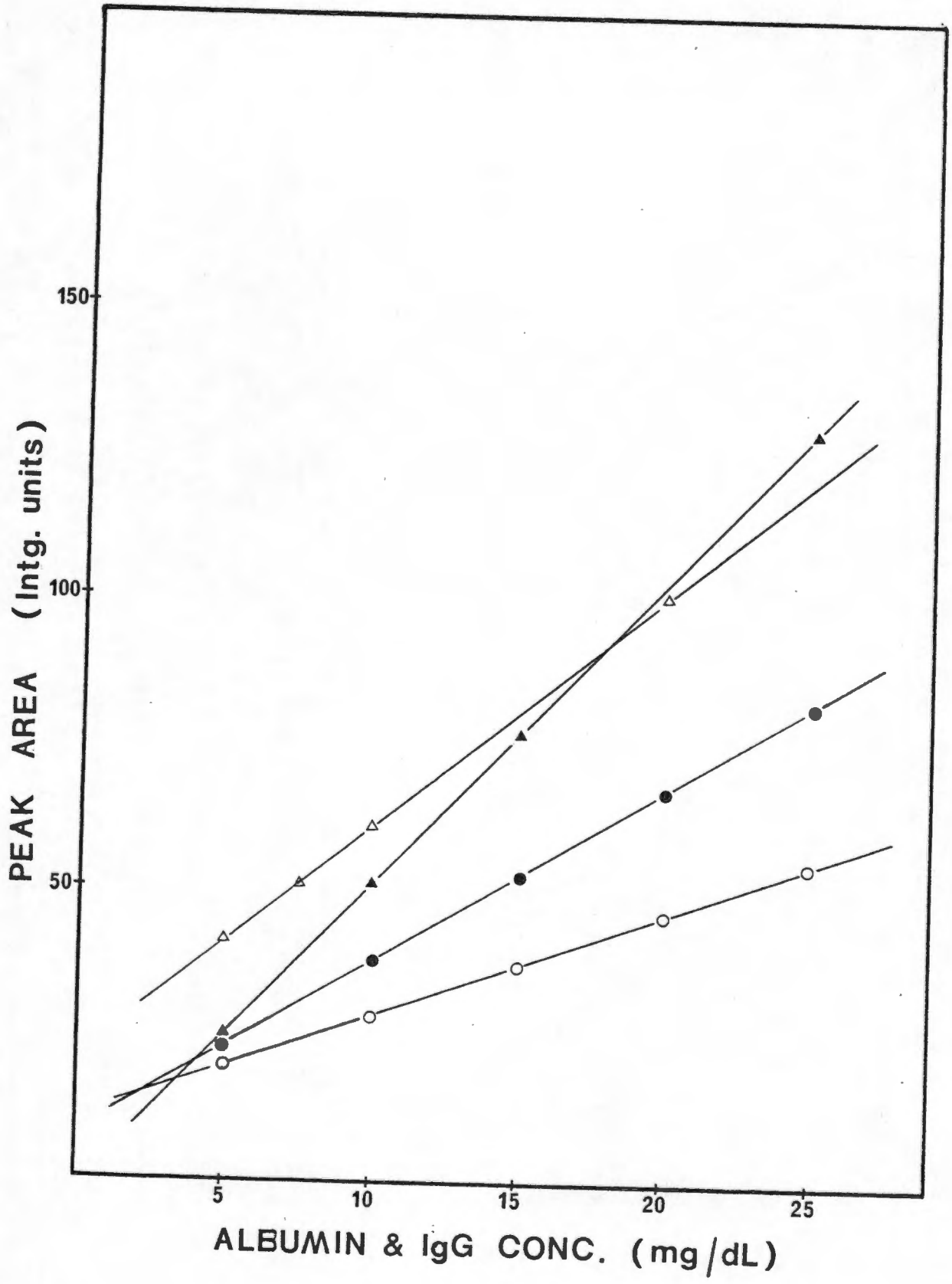

70
60
50
40
30
20
10
PEAK AREA (Intg. units)
5
10
IgG CONC. (mg / dL)

FIGURE 5.9

Comparison between the linear relationships of albumin (●) and IgG (▲) as determined from standard curves and the CSF albumin (○) and CSF IgG (△) as determined by RID.


150
100
50
PEAK AREA (Intg. units)
5
10
15
20
25
ALBUMIN & IgG CONC. (mg/dL)

The standard curve graphs of albumin and IgG are more steeply sloped than those of the CSF results.

It thus appears that the isotachophoretic areas, as obtained from the CSF profile for albumin and IgG, cannot be used in the standard curve graphs, previously constructed, to determine the concentration of these two proteins.

However, a number of points should be noted. Firstly, the standard curve concentrations of albumin and IgG were accurately determined, using the extinction coefficient method. The concentrations of albumin and IgG in the CSF were determined by the inherently less sensitive radial immunodiffusion method. A more sensitive method - e.g. nephelometry, may have given more comparable results. Secondly, the proteins used for the determination of the standard curves were purified commercial products. Thirdly, the electrolyte system used to separate the CSF proteins is not optimal. It has been found (See Chapter VI) that a number of other proteins slot into the "albumin" peak. As a result, the area of the albumin peak in the CSF profile is not due only to albumin. This is confirmed by the CSF albumin curve where the albumin concentration as determined from this graph would give higher values than those obtained from the albumin standard curve. Finally, the area is automatically determined by the Hewlett-Packard integrator and there may

be some variability as to where the albumin peak in the CSF profile starts and ends.

5.2.5 Relationship between Increasing Volume and Peak Area

5.2.5.1 Method

Increasing volumes (2,5 µL to 20 µL) of a CSF sample with spacers were injected using a Hamilton syringe into the Tachophor. The peak areas were recorded.

5.2.5.2 Results

Table 5.9 gives the results of the albumin, transferrin, total IgG and total protein peak areas, when increasing volumes of a CSF sample were injected. A good correlation exists in all cases. The total IgG area is the sum of the three regions bounded by the amino acids: valine, leucine and β alanine, while the total protein is the sum of all peaks obtained in the CSF separation pattern.

The computed "best fit" results (Table 5.10) were used to draw the graphs (Figure 5.10). A direct linear relationship exists between volume and the resultant peak area.

TABLE 5.9 - DUPLICATE RESULTS OF INCREASING CSF VOLUME AND PEAK AREA FOR ALBUMIN PEAK, "TRANSFERRIN" PEAK, TOTAL IgG AND TOTAL PROTEIN

CSF VOLUME (µL)	PEAK AREA (Intg. units)			
	ALBUMIN PEAK	"TRANSFERRIN" PEAK	TOTAL IgG [a] PEAKS	TOTAL [b] PROTEIN
2,5	53,2	27,4	58,6	311,4
	47,4	21,7	40,2	241,4
5,0	70,0	32,7	60,2	375,2
	60,8	27,1	52,8	259,7
10,0	90,6	37,0	73,4	461,1
	99,6	42,3	85,0	415,6
15,0	128,0	52,7	93,1	620,9
	135,3	67,6	110,2	725,0
20,0	146,2	68,1	125,0	705,5
	152,1	51,0	137,5	726,5
Correlation Coefficient	0,9895	0,9074	0,9701	0,9569
Y Intercept	36,7	19,2	34,4	193,9
Slope	5,85	2,23	4,67	27,6

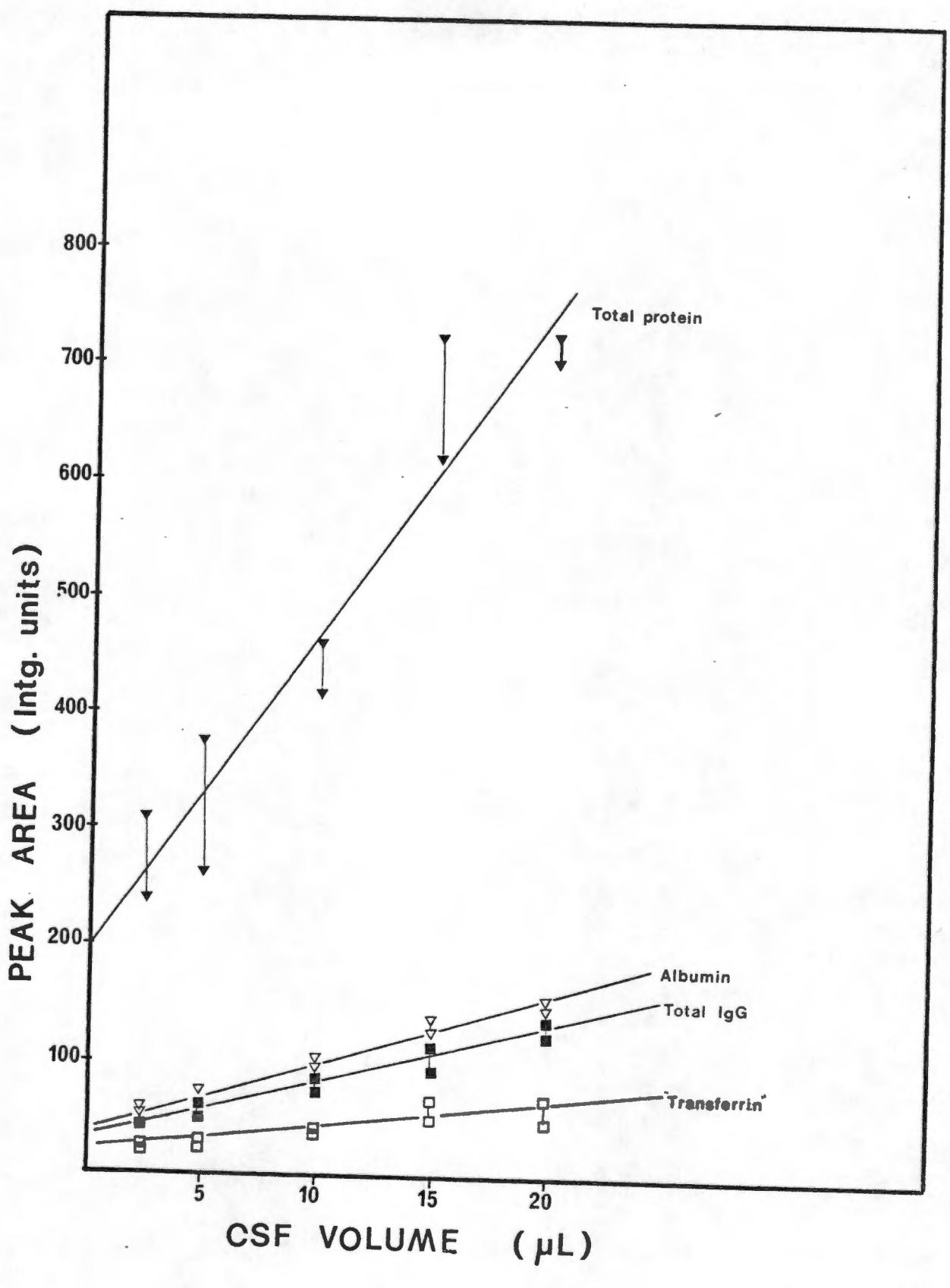
a. Sum of 3 IgG peaks bounded by the amino acid peaks valine, leucine and β alanine.
b. Sum of all peaks in the CSF pattern.

TABLE 5.10 - COMPUTED "BEST FIT" RESULTS OF INCREASING CSF VOLUME AND PEAK AREA FOR ALBUMIN PEAK, "TRANSFERRIN" PEAK, TOTAL IgG AND TOTAL PROTEIN

CSF VOLUME (µL)	PEAK AREA (Intg. Units)			
	ALBUMIN PEAK	"TRANSFERRIN" PEAK	TOTAL IgG PEAKS	TOTAL PROTEIN
2,5	51,3	24,8	46,0	263,0
5,0	66,0	30,4	57,7	332,0
10,0	95,2	41,5	81,1	470,2
15,0	124,5	52,7	104,4	608,3
20,0	153,8	63,8	127,7	746,4

FIGURE 5.10

Relationship between increasing CSF volume and total protein (▼——▼), albumin (▽——▽), total IgG (■——■) and "transferrin" (□——□).


800
700
600
500
400
300
200
100
PEAK AREA (Intg. units)
Total protein
Albumin
Total IgG
Transferrin
5
10
15
20
CSF VOLUME (µL)

5.2.6 Reproducibility Studies

5.2.6.1 Method

10 µL of a CSF sample with spacers was examined six different times under standardized conditions. The areas of some of the UV-absorbing peaks were recorded.

5.2.6.2 Results

The profiles of the six isotachophoretic separations of the CSF sample shows the consistency and reproducibility of the technique (Figure 5.11). The areas of the shaded peaks, the total IgG and total protein were recorded (Table 5.11). The percentage variation for each of the different fractions from the mean area is given (Table 5.12). The variation ranges from 0,2 - 18% with an average of 7,12 $\pm$ 4,8%.

5.3 DISCUSSION

The quantitative analysis of complex protein mixtures by isotachophoresis has been demonstrated. Direct linear relationships exist between the albumin concentration and peak width and peak area (Figure 5.3).

From the albumin standard curve, the zone length equivalent to a concentration below 70 mg/dL does not occupy the full UV detector slit width. The response time of the detector is less than the time taken for the zone to pass the slit and therefore the UV absorption does not reach full span. At

FIGURE 5.11

Isotachopherograms of a single CSF sample examined six different times.

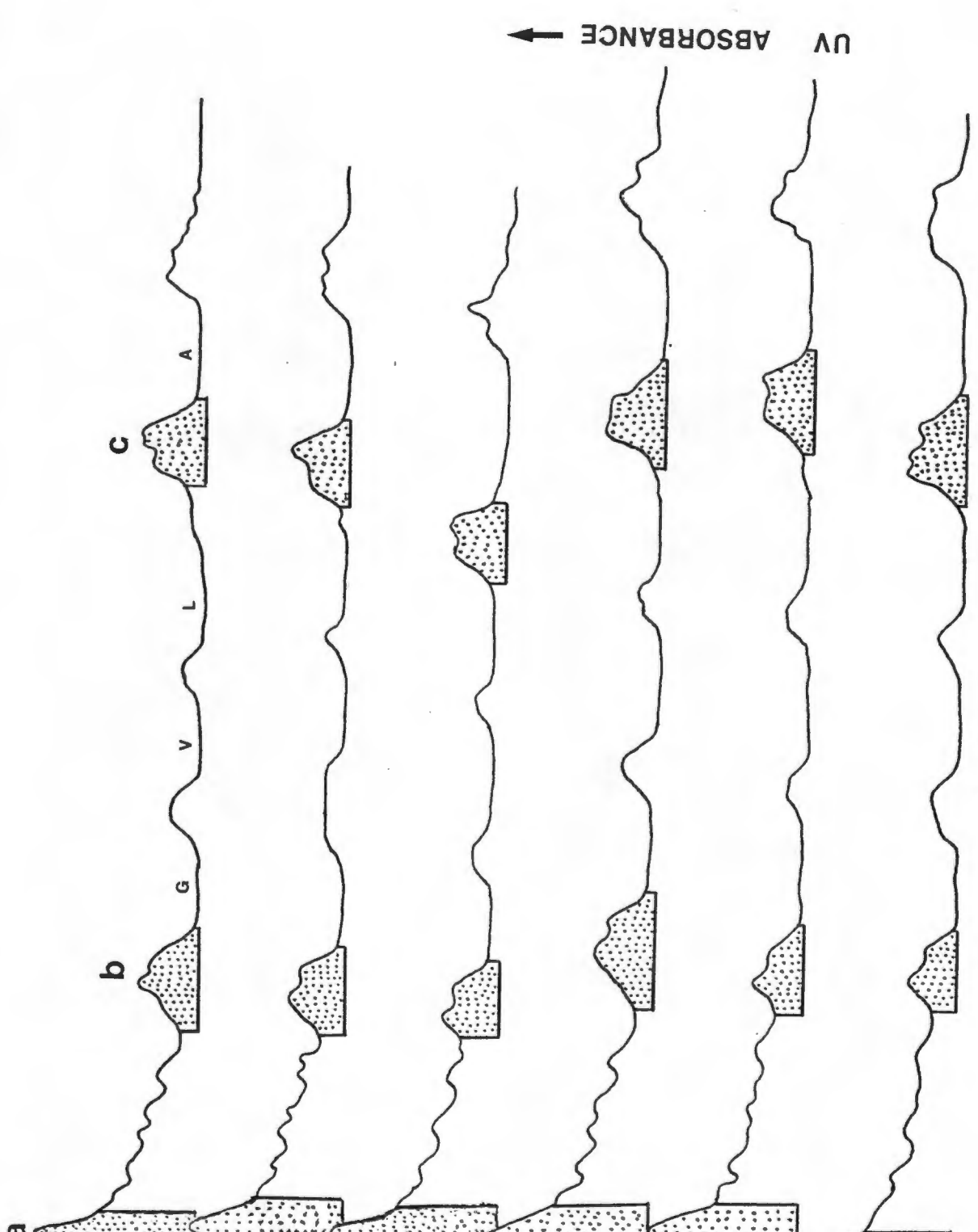

UV ABSORBANCE
a
b
c
G
V
L
A
1
2
3
4
5
6
TIME
30 seconds

TABLE 5.11 - REPRODUCIBILITY STUDIES. A SINGLE CSF EXAMINED 6 TIMES AND PEAK AREAS RECORDED AS INTEGRATED UNITS

PEAK FRACTION	RUN NUMBER					
	1	2	3	4	5	6
ALBUMIN a	109,2	87,0	106,4	89,2	91,5	90,9
TRANSFERRIN b	37,8	44,5	32,6	41,0	36,8	33,8
LEUCINE-ALANINE c	40,9	46,2	45,0	43,3	47,9	45,8
TOTAL IgG	61,2	60,2	68,2	65,7	62,3	60,0
TOTAL PROTEIN	305,7	367,8	348,7	320,5	286,5	366,8

(a, b, c - See Figure 5.11)

TABLE 5.12 - REPRODUCIBILITY STUDIES. THE PERCENTAGE VARIATION OF EACH RUN COMPARED TO THE MEAN FOR THE DIFFERENT FRACTIONS

PEAK FRACTION	RUN NUMBER					
	1	2	3	4	5	6
ALBUMIN a	14,1	10,0	11,1	7,2	4,5	5,2
TRANSFERRIN b	0,2	18,0	15,6	8,7	2,4	11,5
LEUCINE-ALANINE c	9,5	3,1	0,4	3,4	6,9	2,2
TOTAL IgG	2,7	4,4	8,4	4,4	0,9	4,8
TOTAL PROTEIN	8,8	10,5	4,8	3,7	16,0	10,2

(a, b, c - See Figure 5.11)

these low concentrations the peak height may be used as a quantitative measure.

The concentrations of albumin at which the zone length may be accurately measured is greater than the CSF albumin concentration. Thus, the peak height or the accurate peak area measurement is a more acceptable and useful parameter.The peak area measurements also show a higher correlation than the two other parameters - 0,9967 compared to 0,9731 and 0,9561.

As the immunoglobulin region is artificially divided into three sub-fractions by the amino acid spacers, the peak width and peak height could not be used as an accurate method of quantitation. However, the correlation coefficient between IgG concentration and total peak area is high - 0,9837.

The addition of spacers to the standard curve protein mixtures is necessary if direct readings from the CSF pattern (which contains these spacers) are to be made. The ampholytes tend to widen the peaks, as their mobility range is such that they often separate with a protein.

Comparison of the albumin and IgG standard curves with those obtained from the CSF show little similarity. This may, in part, be due to the relatively inaccurate method used to quantitate CSF albumin and IgG levels, namely radial immunodiffusion. Probably if the more sensitive methods of immunonephelometry (Savory et al., 1972; Killingsworth and Savory,

1973; Kjellin and Hallander, 1980), radioimmunoassay (Nerenberg and Prasad, 1975; Nerenberg et al., 1978; Mingioli et al., 1978), fluorimmunoassay (Stevens et al., 1979) or enzyme immunoassay (Kobatake et al., 1980) were used, a more satisfactory correlation may have been found. However, the apparatus necessary for these techniques was not available.

It should also be noted that the "albumin" peak in the CSF pattern contains other proteins - e.g. prealbumin, orosomucoid and $\alpha_1$anti-trypsin, which do not separate under the conditions used. (See Chapter VI)

The accuracy of the quantitative determinations depends on a number of precautions being taken in thermostating the Tachophor, ensuring constancy of current density (Everaerts et al., 1973) and injecting the same sample volume. Variations in these parameters and the concentration of the leading electrolyte will result in different zone lengths for the same amount of sample (Everaerts et al., 1973).

The position at which the sample is injected is also of importance (Holloway and Lustorff, 1980). The use of an injection block (Everaerts and Verheggen, 1970b) ensures that the sample is introduced into the system at the same position. In the present study, the sample was introduced 2 mm into the leading electrolyte and reproducible results were obtained.

Quantitative studies using isotachophoresis have been done on a wide variety of substances. The levels of methotrexate (Driessen et al., 1980), theophylline (Moberg and Hjalmarsson, 1980; Reijenja et al., 1984), 5-fluorouracil (Gustavsson et al., 1979, 1983) in the sera of patients treated with these drugs have been measured. It is also possible to separate and quantitate penicillin and tetracycline antibiotics (LKB Incentive Group, 1977).

A number of metabolic disorders have been examined and quantitative studies performed. High levels of isovalerylglycine are excreted in the urine of patients with isovaleric acidemia (Kodama 1979); phenylalanine concentration in phenylketonuric sera could be accurately quantitated (Kopwillem et al., 1974); prolidase activity in erythrocytes of patients with iminopeptiduria could be measured (Mikasa et al., 1984); quantitative analysis of cystathionine and perhydro 1,4-thiazepine 3,5 dicarboxylic acid in urine of patients with cystathioninuria (Kodama et al., 1984); abnormal levels of aspartic acid, asparagine, glutamic acid and glutamine occur in children with metabolic disorders affecting the nervous system, and a good correlation between concentration as determined isotachophoretically and by ion exchange chromatography was found (Robinson and Rimpler, 1978).

The tissue metabolites (ATP, AMP and ADP) were accurately quantitated from muscle (Sjodin et al., 1974, 1975) and in mouse liver cells (Dunn and Kemp, 1974). Beckers and Everaerts (1972b) successfully separated and quantitated nucleotides.

A variety of purine and pyrimidine inborn metabolic error disorders have been characterized by the characteristic metabolite excreted and quantitative studies perfomed on sera from patients with hypoxanthanine guanine phosphoribosyl transferase deficiences (Oerlemans et al., 1979b) and on urine from patients with xanthuria, adenosine deaminase and adenine phosphoribosyl transferase deficiency (Simmonds et al., 1979).

Isotachophoresis has been found to be a rapid technique, compared to gas chromatography, in the quantitation of mandelic acid, phenylglyoxylic acid, hippuric acid and methylhippuric acid in the urine after occupational exposure to styrene, toluene and/or xylene (Sollenberg and Baldesten, 1977).

Quantitation of uric acid in the serum by isotachophoresis correlated well with the standardized enzymatic methods and the results were not influenced by natually occurring metabolites and drugs which affect the latter method (Oerlemans et al., 1979a; Verheggen et al., 1980).

Uremic blood samples from pre- and post-dialysis sera were analyzed for the presence of toxins and lactate, phosphate and acetate could be quantitated by isotachophresis (Mikkers et al., 1979c, 1979d). The oxalate and citrate concentration in urine could also be determined (Schmidt et al., 1979; Tschöpe and Ritz, 1980, Tschöpe et al., 1981).

The purity and quantity of synthetic peptides, undecapeptide and decapeptide (Kopwillem et al., 1975) and human growth hormone (Kopwillem et al., 1973) has been assessed and measured by isotachophoresis. Miyazaki and Katoh (1976) measured the concentration of a variety of peptides - e.g. bradykinin and oxidized and reduced forms of glutathione, using a potentiometric detector.

The concentration of anions and cations in natural fruit juices was measured, using a Tachophor equipped with a conductivity detector (Everaerts et al., 1974). The conductivity detector was also used in the determination of conjugated bile acids in human bile (Reijenga et al., 1983b).

Isotachophoresis has also been used to measure ortho- and pyro-phosphates in liquid fertilizers (Boček et al., 1978b) and lactic and acetic acids in silage extracts (Boček et al., 1978c), with the latter giving an indication as to the quality of silage.

This list illustrates the range of substances that can be quantitated using isotachophoresis.

Finally, the reproducibility in the present study of the technique was found to be better than 7% which compares favourably with Delmotte's (1977) figure of under 10% and Gustavsson et al., (1983) of 7-8% at low concentrations and 4-5% at high concentrations. These values are greater than those obtained by Kodama (1979) of 2,15%; Miyazaki and Katoh (1976) of 1,65%; Boček et al. (1978c) of 2,3% and Oerlemans et al. (1981) of 2%. The reason is that the latter authors were examining small molecular weight molecules and did not require spacers, especially ampholytes, for separation. Isotachophoresis is ideally suited to the examination of small molecules but gives very satisfactory results for the larger molecules - e.g. proteins.

CHAPTER VI

IDENTIFICATION OF THE CSF UV-ABSORBING PEAKS AS SHOWN IN THE ISOTACHOPHEROGRAM

6.1 INTRODUCTION

The isotachopherogram of CSF shows two main regions of UV-absorbing peaks. The front running peak (FRP) region consists of peaks of mobility greater than albumin. The second region contains the main proteins found in CSF and is artificially spaced with amino acids and ampholytes to aid in interpretation.

The identification of these peaks is difficult. Various methods may be employed, of which the most direct and simple one is the addition of specific substances to the CSF and observing resulting peak increments on the isotachopherogram. However, a major problem with these "doping" experiments is obtaining proteins in a sufficiently pure form. Contaminating substances have often been found in these protein preparations (details given below).

An elegant method for collecting and identifying separated components is the fraction collector device first developed by Arlinger (1976) and subsequently refined and made commercially

available as the LKB 2127 Tachofrac (LKB - Produkter AB, Bromma, Sweden). Separated proteins are collected on a slowly moving cellulose acetate strip, after passing the UV detector. The strip with the collected fractions may then be subjected to immunological, radioactivity and zymogram techniques for further identification. Many workers have successfully used this technique for the identification of serum and CSF proteins (Arlinger, 1976; Hjalmarsson, 1977; Kjellin and Siden, 1978; Kjellin and Hallander, 1979a).

In this chapter, a description of the identification some of the peaks in the main CSF region were determined by "doping" experiments.

The identity of the FRP normally recorded in the CSF isotachopherogram, as well as an abnormal component found in viral meningitis, was investigated, using a variety of techniques. These included physiochemical and chromatographic methods.

6.2 IDENTIFICATION OF NORMAL CSF PROTEINS

6.2.1 Method

UV-absorbing peaks recorded in the isotachopherogram of the main region of CSF protein profile were identified by the addition of relatively pure proteins to the CSF and observing a resultant peak increase.

9 µL of CSF with spacers was injected into the capillary, together with 1 µL of a specific protein solution (usually 10 mg/mL in physiological saline). The MES/Ammediol leading electrolyte system was used.

The proteins examined were human albumin, human transferrin, human glycoprotein and human IgG, all obtained from Sigma Chemical Co., U.S.A. Prealbumin, haptoglobin, α_1 antitrypsin, α_2 macroglobulin, IgA and monoclonal and polyclonal IgM were all from Calbiochem - Behring Corp., U.S.A.

6.2.2 Results

The results of the doping experiments are given in Figure 6.1.

The addition of albumin resulted in the first large peak in the main CSF separation profile being increased in height and width (Figure 6.1b).

The addition of glycoprotein resulted in the "albumin" peak being increased (Figure 6.1c). Prealbumin, orosomucoid and α_1 anti-trypsin also increase the same peak (results not shown). Thus, under the electrolyte conditions used in this present study, these proteins could not be separated and identified. It would be necessary to change the electrolytes to a lower pH in order to separate these proteins.

FIGURE 6.1

Identification of normal CSF proteins using "doping" experiments

(a) normal CSF protein profile. To this CSF was added a number of different proteins

(b) 10 μg human albumin

(c) human glycoprotein, human prealbumin, human orosomucoid and human α_1 anti-trypsin

(d) 10 μg human haptoglobin

(e) 1 μg human transferrin (.....), 10 μg transferrin (——)

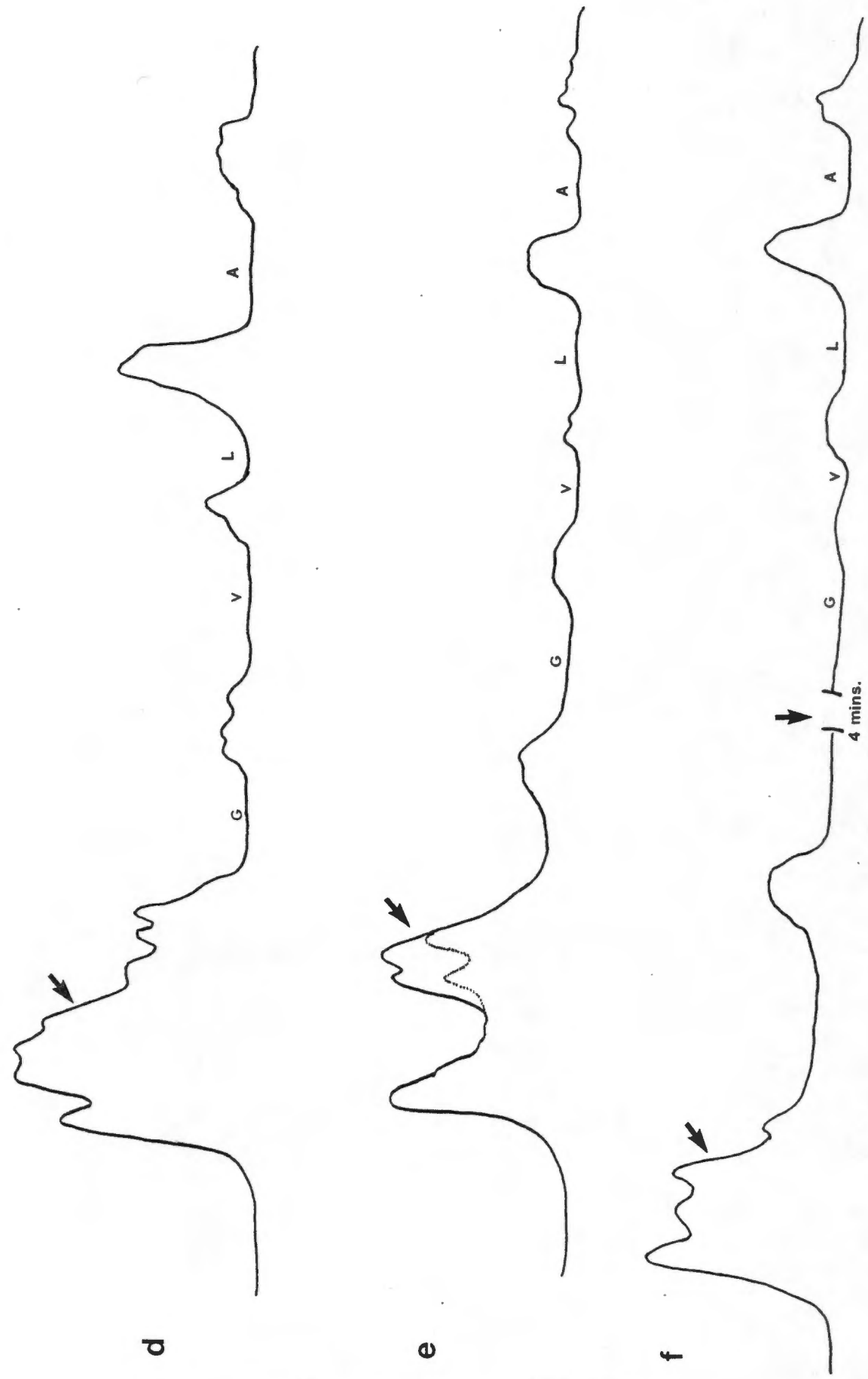
(f) 10 μg human α_2 macroglobulin

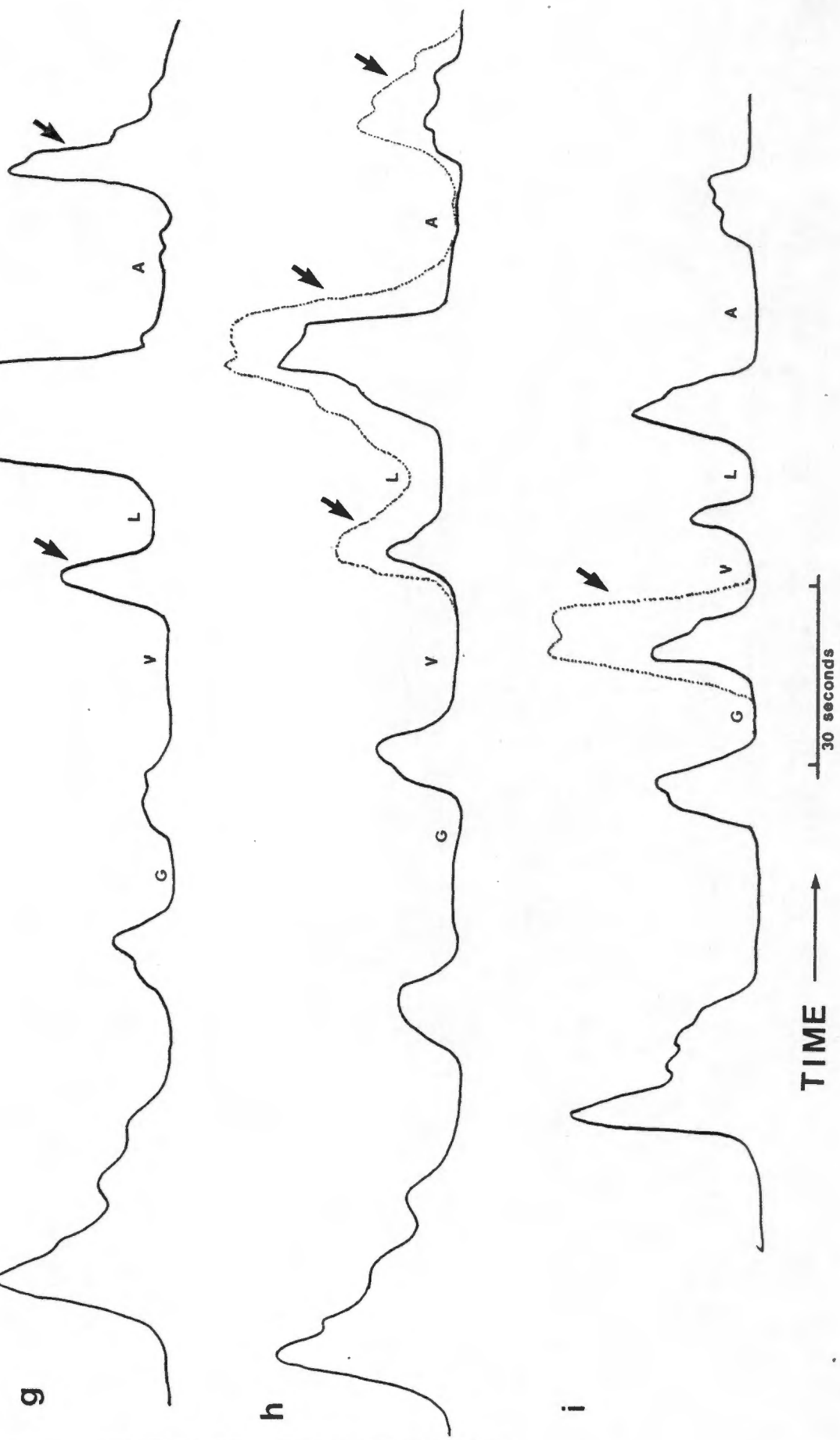
(g) 10 μg human IgG

(h) polyclonal IgM (.....), monoclonal IgM (———)

(i) human IgA.

ABSORBANCE 280 nm
c
b
a
TIME
30 seconds
G
V
L
A
G
V
L
A
G
V
L
A


ABSORBANCE 280 nm
TIME
30 seconds
4 mins.
G
V
L
A
d
e
f


ABSORBANCE 280 nm
g
h
i
G
V
L
A
TIME
30 seconds

Haptoglobin appears as a peak immediately behind the albumin peak and in the same position as α_2 macroglobulin (Figure 6.1d).

Transferrin was diluted to 1 mg/mL to determine the position of this protein more accurately in the CSF profile. The peak after the albumin peak was increased (Figure 6.1e).

The addition of α_2 macroglobulin resulted in two areas of the CSF pattern being affected. Firstly, two peaks of mobility less than the albumin peak were increased. A long non-UV absorbing region is seen in the same position as glycine (Figure 6.1f).

The IgG region is artificially spaced with the amino acids valine, leucine and β alanine. The addition of IgG resulted in all three regions being increased with a predominant increase between the amino acid spacers, leucine and β alanine (Figure 6.1g).

Both monoclonal and polyclonal IgM increased the three IgG peaks. Thus, IgM separates together with the IgG and they cannot be distinguished from each other (Figure 6.1h).

IgA occurs between the amino acid spacers, glycine and valine. Some non-UV material present in the IgA preparation split off some of the peaks that are normally attached to the albumin peak (Figure 6.1i).

6.3 INVESTIGATIONS TO IDENTIFY THE FRONT RUNNING PEAKS

Some of the physical properties of the FRP were determined. The approximate molecular size was determined by dialysis experiments. This method was also used to investigate the effect of different sodium chloride (NaCl) concentrations on these FRP.

The effect of a higher mobility leading ion, the chloride ion, was determined.

A variety of low molecular weight substances were added to the CSF to determine if these substances would increase one of these FRP.

6.3.1 Dialysis Experiments

6.3.1.1 Method

0,5 mL samples of a single CSF sample were dialyzed overnight (18 hrs) at 4°C against (1) physiological saline (0.9%); (2) 0,45% (m/v) saline; and (3) distilled water. Dialysis tubing (flat width 10 mm) of two different molecular weight (MW) cutoff pore sizes were used in each

experiment, namely 1000 MW and 12-14000 MW (Spectrum Medical Industries, Inc., Los Angeles, U.S.A.).

The dialyzed samples (10 μL) with spacers were examined in the MES/Ammediol leading electrolyte system. This two-fold experiment investigated both the effect of salt on the FRP, as well as indicating the molecular size of the substances present in the FRP.

6.3.1.2 Results

From the isotachopherograms (Figure 6.2), a loss of the FRP is observed when the CSF was dialyzed against physiological saline. This occurred when both the large and small pore size dialysis membrane tubing were used. Thus, it may be concluded that the FRP have a molecular size of less than 1000 daltons.

The second part of the experiment examined the effect of reducing salt concentration on the FRP. There is a gradual reduction of the FRP as the NaCl concentration is reduced (Figure 6.2). This is due to the components in this region migrating zone electrophoretically in an "out of stack" configuration in the absence of the chloride ion and, therefore, not concentrating. Detection of these components is thus difficult.

FIGURE 6.2

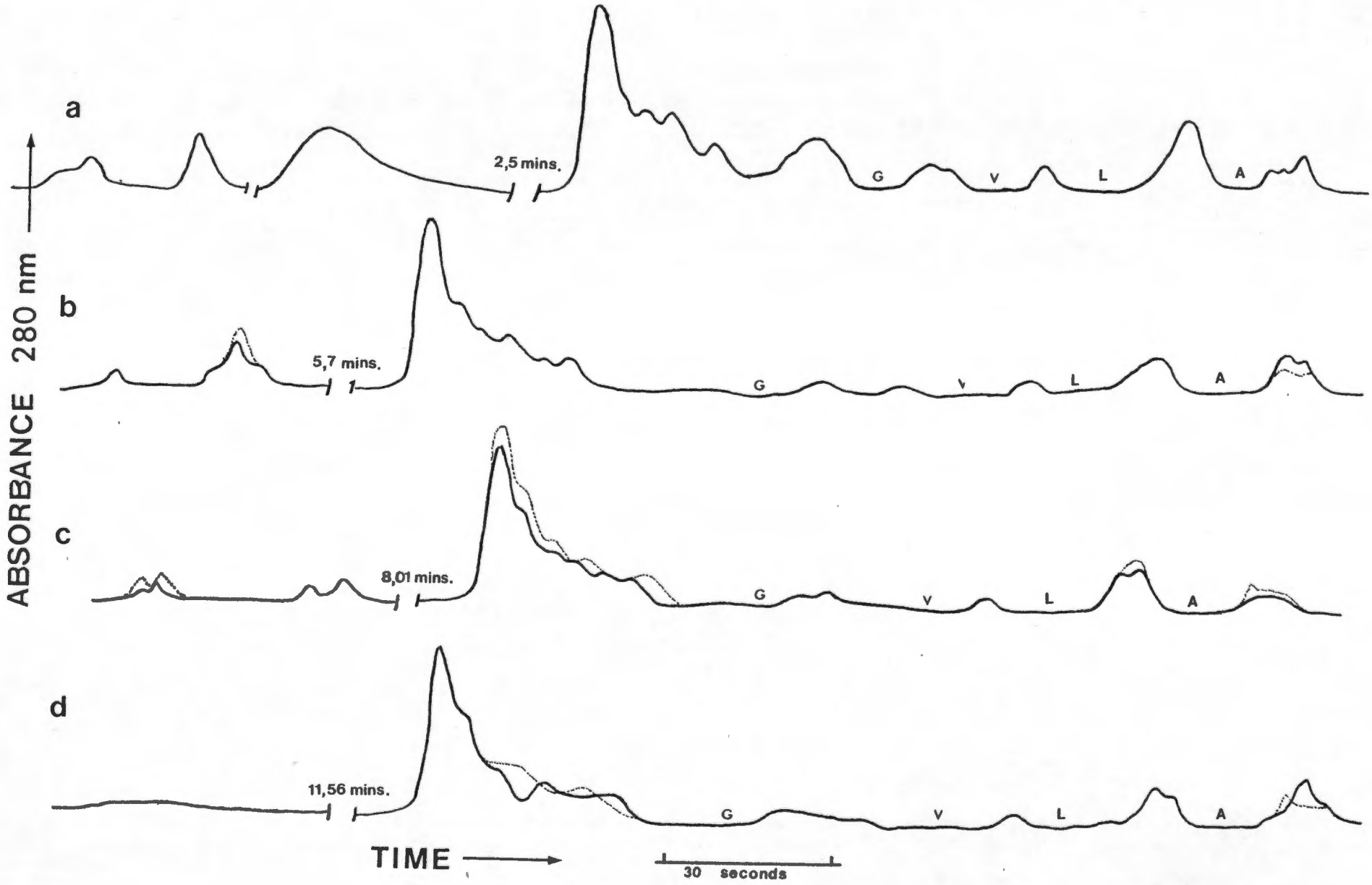
Effect of dialysis of CSF on the protein profile and FRP region.

(a) normal CSF protein profile

(b) CSF dialyzed in 12000 MW (———) and 1000 MW (.....) cutoff dialysis membrane tubing, against physiological saline

(c) CSF dialyzed in the two different molecular weight cutoff tubings against 0,45% NaCl

(d) CSF dialyzed in the two different molecular weight cutoff tubings against distilled water.


a
2,5 mins.
G
V
L
A
b
5,7 mins.
G
V
L
A
c
8,01 mins.
G
V
L
A
d
11,56 mins.
G
V
L
A
ABSORBANCE 280 nm
TIME
30 seconds

The rest of the CSF pattern remained relatively constant, although the peak of mobility greater than the amino acid spacer glycine was lost (Figure 6.2). The IgG peak between the spacers leucine and β alanine is reduced, probably due to adsorption to the dialysis membrane.

Another feature that should be noted is the change in position of the FRP in relation to the albumin peak. As the salt concentration is reduced, the FRP migrated further ahead of albumin.

6.3.2 HCl/Ammediol Leading Electrolyte Experiments

The effect of changing the leading ion to one of a higher mobility was investigated in Chapter IV, section 4.2.3. The chloride ion was used instead of MES. The results showed that the FRP have mobilities greater than the MES ion, but less than the chloride ion.

6.3.3 Addition of Low Molecular Weight Substances to the CSF Sample

As it has been shown that the FRP have a molecular weight less than 1000 daltons, a variety of low molecular weight substances were added to the CSF to see if any of the FRP could be increased. The substances included bovine bilirubin, nicotinamide adenosine dinucleotide (NAD), adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). These substances separated in the

FRP region but did not specifically increase any one of the three FRP.

On routine analysis of a CSF sample, an abnormal pattern for the FRP region was observed (Figure 6.3). It was subsequently established that the patient had been treated intrathecally with methotrexate, a drug used in the treatment of leukaemic patients. This drug with the chemical description, L-(+)-N-(p((2,4 diamino-6-pteridinyl) methyl methylamino) benzoyl) glutamic acid, is a folic acid antagonist and has a chemical structure similar to that of folic acid (Figure 6.4). The full chemical description of folic acid, or more correctly, pterylglutamic acid is N-(4-(((2-amino-1,4-dihydro-4-oxo-6-pteridinyl) methyl) amino) benzoyl)-L-glutamic acid.

As methotrexate separated ahead of albumin in the FRP region, it was anticipated that folic acid (MW 441,4) a normal constituent of CSF, would also occur in this region. It occurs in the CSF at a concentration of 15 - 50 ng/mL (Giegy Scientific Tables; Chanarin, 1980).

Another normal constituent of the CSF is uric acid (MW 168,11) with a concentration of 0,25 mg/dL (Fishman, 1980). The chemical structure is given in Figure 6.5 and is compared with a similar substance, xanthine (MW 152,1).

FIGURE 6.3

Analysis of CSF sample from a patient treated intrathecally with methotrexate.

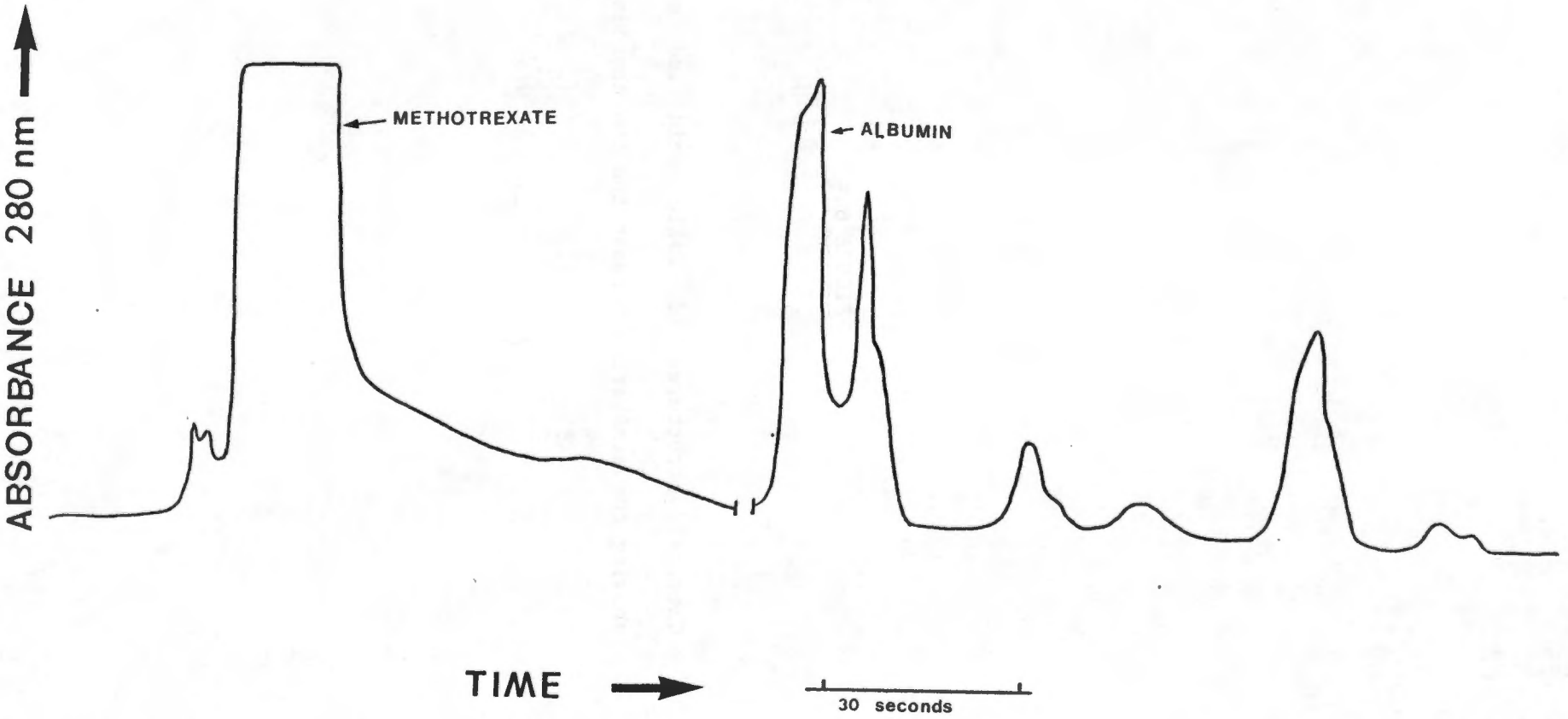
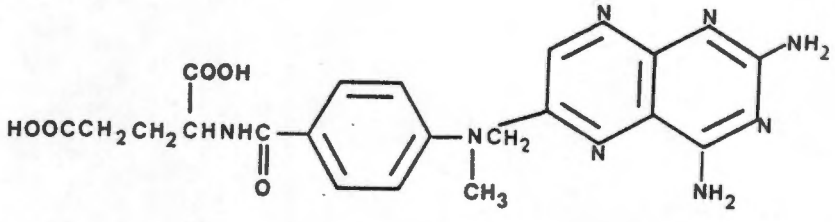
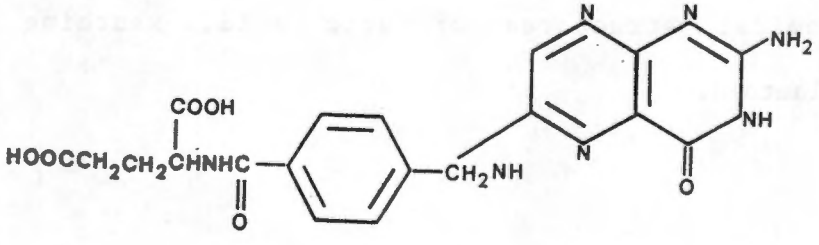

ABSORBANCE 280 nm
METHOTREXATE
ALBUMIN
TIME
30 seconds

FIGURE 6.4

Chemical structures of folic acid and methotrexate showing the similarity between the two compounds.



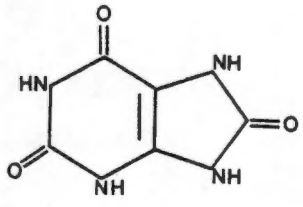
Methotrexate



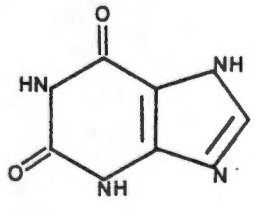
Folic acid

FIGURE 6.5

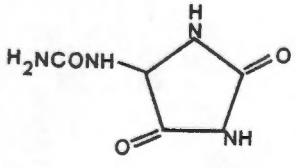
Chemical structures of uric acid, xanthine and allantoin.



Uric acid



Xanthine



Allantoin

6.3.3.1 Methods

1 µL of a saturated aqueous solution of folic acid (Sigma Chemical Co., U.S.A.) was injected, together with 9 µL CSF. The MES/Ammediol leading electrolyte system was used.

1 µL of a saturated aqueous solution of uric acid (Merck, Darmstadt) was injected, together with 9 µL CSF and examined in the MES/Ammediol leading electrolyte system.

1 µL of a solution of xanthine (10 mg/dL) (National Biochemicals Corp., Ohio, U.S.A.) was also injected with 9 µL CSF and the results recorded.

A CSF with a greatly elevated peak in the FRP region was treated with the enzyme uricase. 60 µL CSF was incubated at 37°C for 30 minutes with 1,5 µL (2 mg/mL) uricase. The pre- and post-treated CSF samples were separated in the MES/Ammediol leading electrolyte system and the results recorded.

1 µL allantoin (Figure 6.5) (1 mg/mL), the breakdown product of uric acid and the enzyme (1 µL) were also specifically added to the CSF to determine their positions in the CSF separation profile.

6.3.3.2 Results

The first of the FRP (FRP 1) was increased considerably when folic acid was added to the CSF (Figure 6.6b). From this "doping" experiment it would appear that the first peak in this region is folic acid.

The addition of uric acid substantially increased the third peak in the FRP region. By increasing the volume, injected, this peak could be further increased. The zone in the MES/Ammediol system is large and broad (Figure 6.6c).

Xanthine, a molecule of a very similar structure to uric acid, does not occur in the same position, although it also appears in the FRP region (Figure 6.6d).

The identity of the component comprising FRP 3 as uric acid was confirmed when a CSF with an enlarged peak in this position was incubated together with uricase. The large peak in the FRP region was considerably reduced and two changes to the CSF pattern were noted (Figure 6.7). Firstly, an extra UV-absorbing peak appeared which was confirmed to be the enzyme when more of the enzyme was added to the CSF (Figure 6.8b). A non-UV absorbing zone also appeared and, by doping experiments with allantoin, was proved to be the breakdown product of uric acid (Figure 6.8a).

FIGURE 6.6

Identification of FRP

(a) control CSF sample

(b) CSF with 1 μL saturated aqueous solution of folic acid

(c) CSF with 1 μL saturated aqueous solution of uric acid

(d) CSF with 1 μL aqueous solution (10 mg/mL) xanthine.

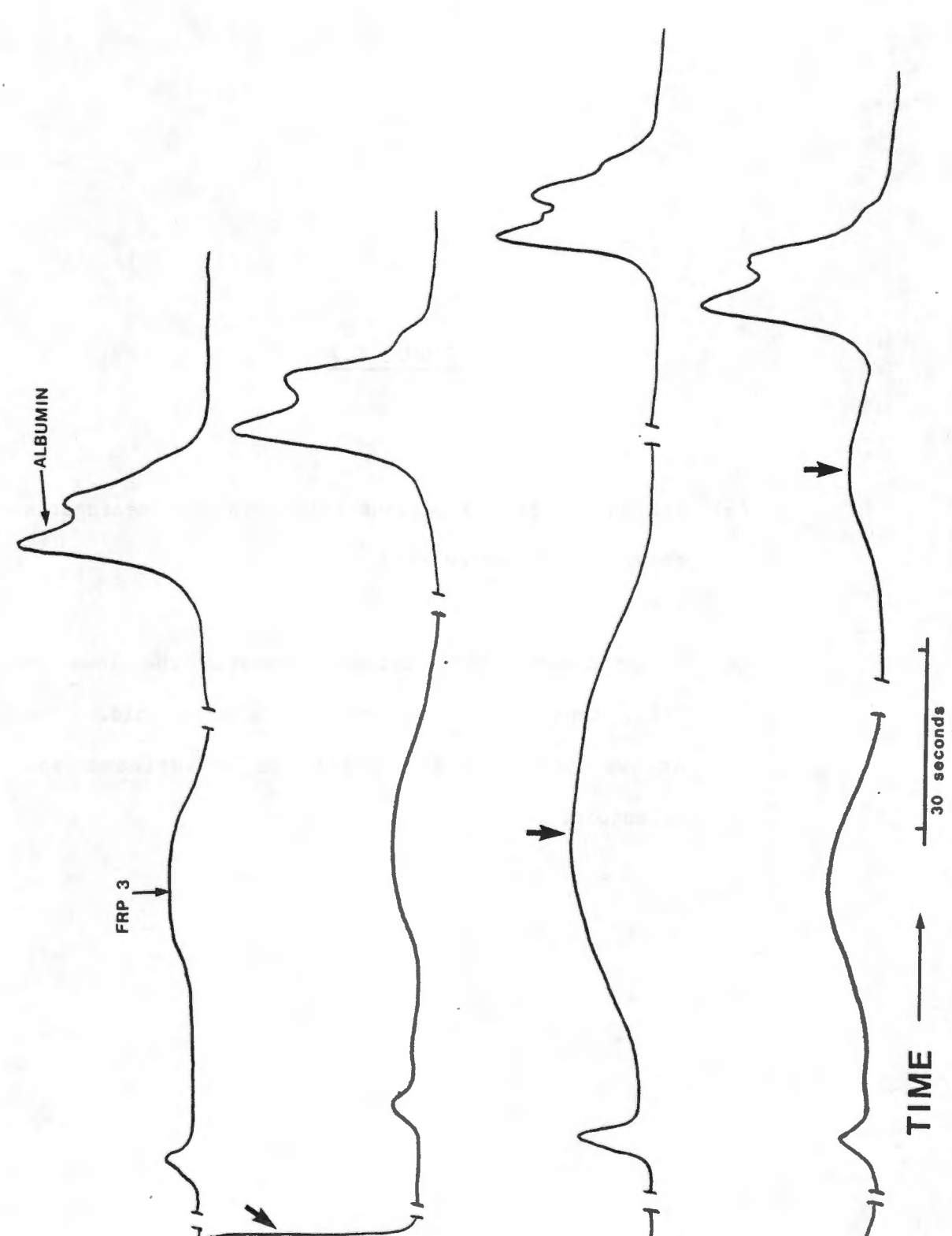

ALBUMIN
FRP 3
FRP 1
a
b
c
d
ABSORBANCE 280 nm
TIME
30 seconds

FIGURE 6.7

(a) CSF sample from a patient with suspected meningitis showing an enlarged FRP3.

(b) CSF incubated with uricase, showing the loss of FRP3, thus confirming this to be uric acid. The arrows indicate the positions of uricase and allantoin.

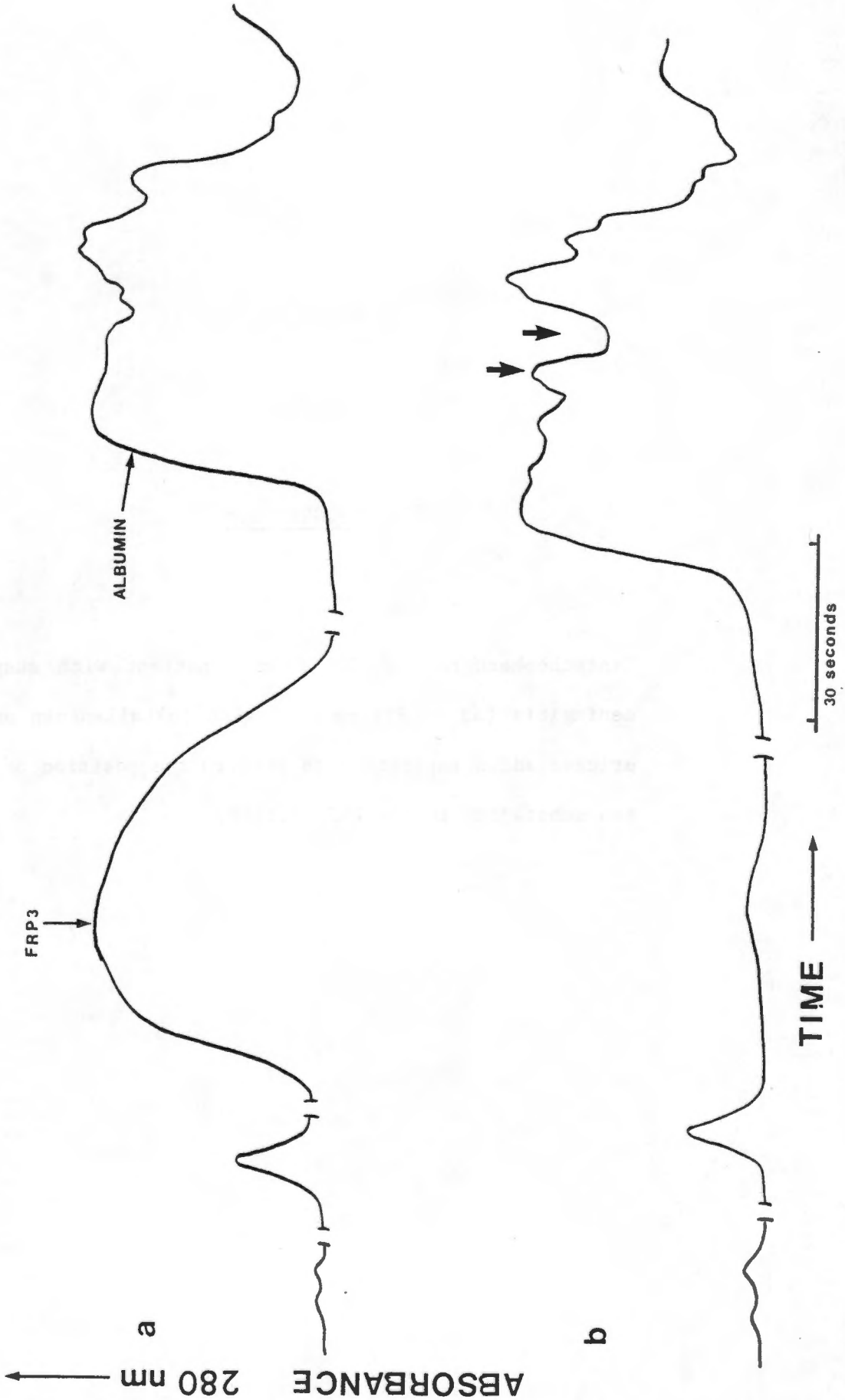
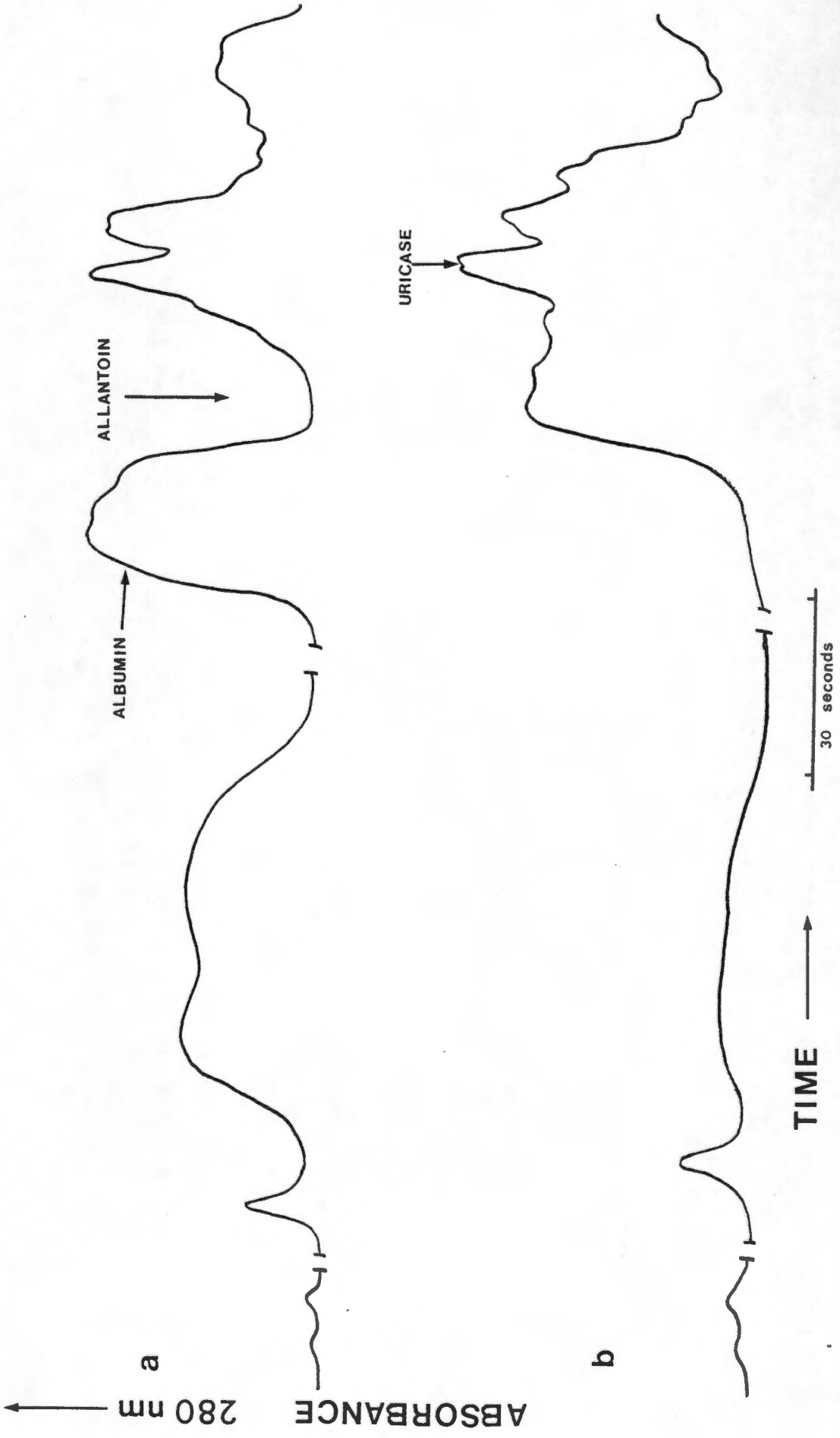

ABSORBANCE 280 nm
a
FRP3
ALBUMIN
b
TIME
30 seconds

FIGURE 6.8

Isotachopherogram of CSF from a patient with suspected meningitis (as in Figure 6.8) with (a) allantoin and (b) uricase added separately to confirm the position of these two substances in the CSF profile.


ABSORBANCE 280 nm
a
ALBUMIN
ALLANTOIN
b
URICASE
TIME
30 seconds

6.4 INVESTIGATION TO IDENTIFY AN ELEVATED COMPONENT FOUND IN SOME VIRAL MENINGITIS CSF SAMPLES

An elevated peak of unknown identity was found in 39% of the viral meningitis CSF samples examined by isotachophoresis (See Chapter VII). The peak increase lies betwen the amino acid spacers, valine and leucine. As IgM is the first immunoglobulin to be produced during an infection, experiments were designed to see if this elevated peak was IgM.

6.4.1 Addition of Human IgM to a CSF Sample

From "Identification of Normal CSF Proteins", Section 6.2, both monoclonal and polyclonal IgM when added to the CSF did not specifically increase the peak between valine and leucine (See Figure 6.1h).

6.4.2 Adsorption of IgM from a CSF Sample

6.4.2.1 Method

A CSF sample with an elevated peak between the spacers valine and leucine was incubated overnight at 20°C with a goat antihuman-IgM coated bead obtained from the HAVAB-M RIA kit (Abbots Laboratory, U.S.A.). 0,2 mL of an undiluted CSF sample was treated in this manner.

10 µL of the treated and untreated CSF with spacers was then examined isotachophoretically.

6.4.2.2 Results

No reduction in the elevated peak was noted with the CSF sample incubated with the anti-IgM coated bead (Figure 6.9).

The area of the peak of interest varied by 3,7% between the untreated and treated CSF sample. This is within the reproducibility range.

6.4.3 Treatment of CSF Sample with 2-Mercaptoethanol

A CSF sample with an elevated peak was treated with an IgM denaturing agent, 2-mercaptoethanol.

6.4.3.1 Method

100 µL CSF was incubated at 37°C for one hour with 33 µL 2-mercaptoethanol (BDH Chemicals Ltd., England) previously diluted 1 : 25 with phosphate buffered saline.

The treated and untreated CSF samples with spacers were then examined by isotachophoresis.

6.4.3.2 Results

2-mercaptoethanol had no effect on reducing the elevated peak (Figure 6.10). The variation between the control and treated CSF was 5% which is within the reproducibility range and, thus, not significant. The denaturing agent slots into the CSF profile as a non-UV absorbing zone.

FIGURE 6.9

Isotachopherogram of a control CSF (a) with an enlarged component between the amino acid spacers valine and leucine and the same CSF (b) after incubation with an anti-IgM coated bead.

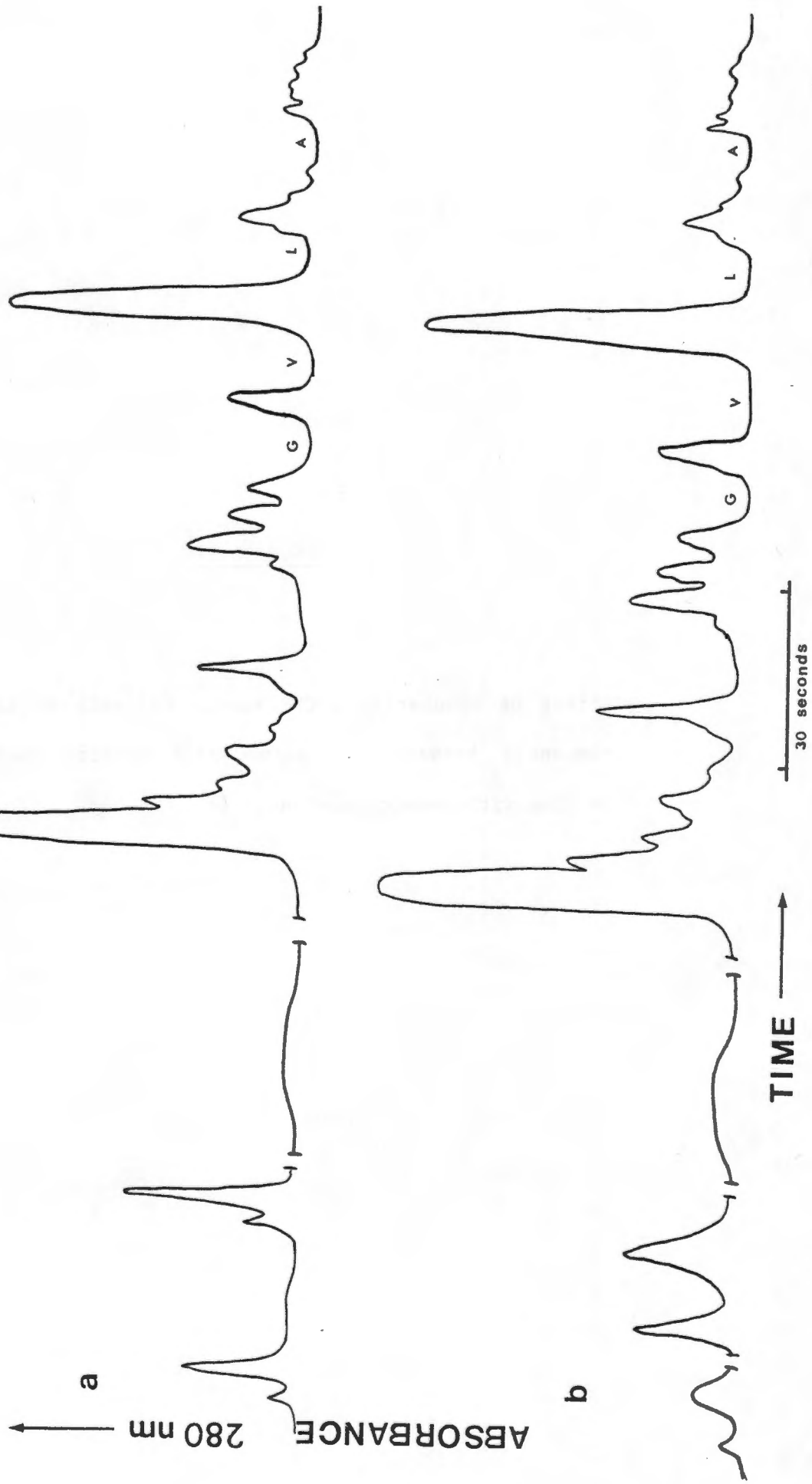
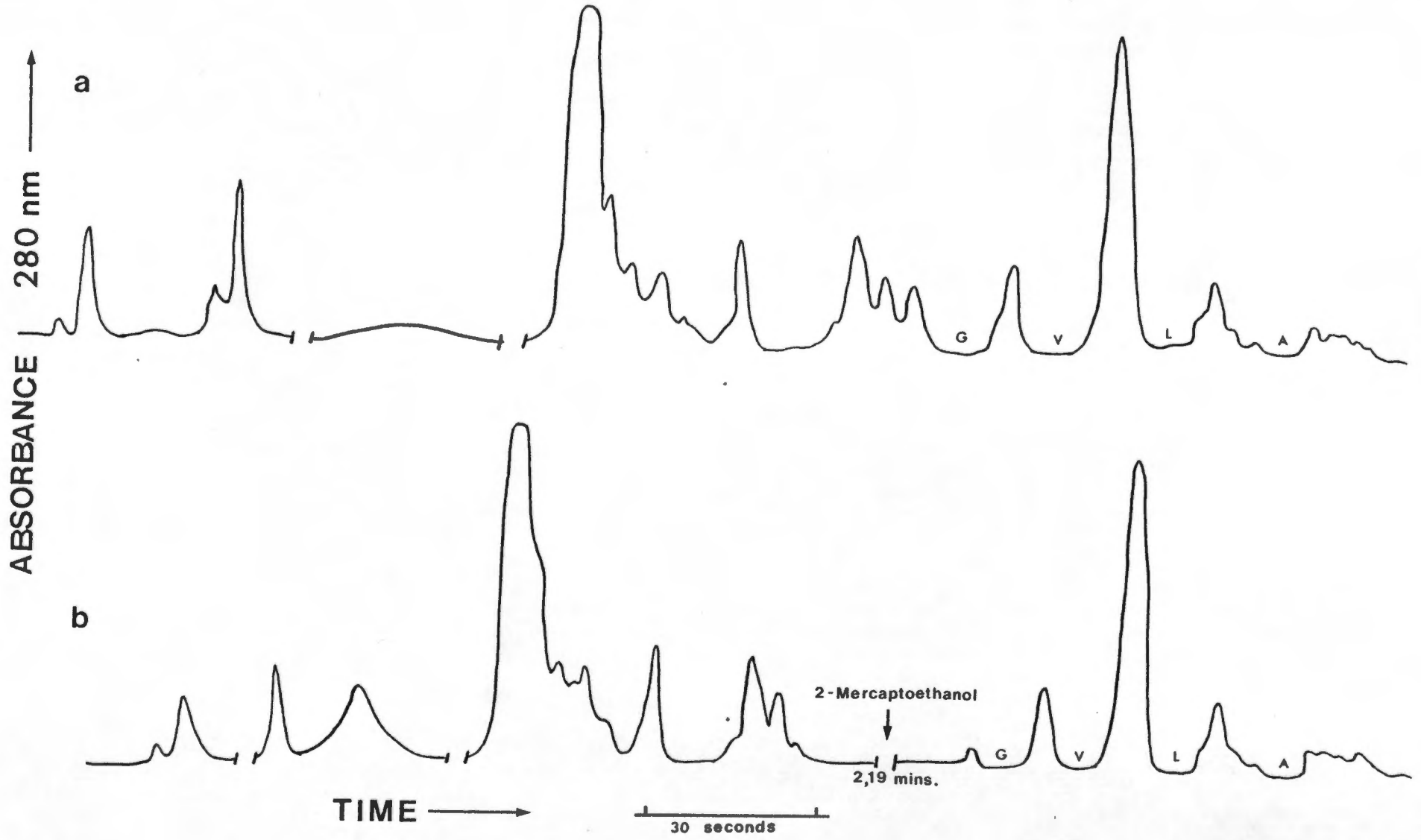

a
b
ABSORBANCE 280 nm
TIME
30 seconds
G
V
L
A

FIGURE 6.10

Effect of incubating a CSF sample (a) with an increased component between the amino acid spacers valine and leucine with 2-mercaptoethanol (b).


a
b
280 nm
ABSORBANCE
G
V
L
A
2-Mercaptoethanol
2,19 mins.
TIME
30 seconds

6.4.4 Protein A Sepharose Chromatography

Protein A, isolated from Staphylococcus aureus, specifically binds with IgG-type antibodies. But the Protein A reactivity is confined to the IgG_1, IgG_2 and IgG_4 subclasses of human IgG (Kronvall and Williams, 1969).

Protein A-Sepharose CL-4B is protein A covalently coupled to a cross-linked matrix, Sepharose CL-4B, by the cyanogen bromide method. This matrix was used to determine the specificity of the component found in some viral meningitis samples.

6.4.4.1 Method

250 mg Protein A-Sepharose CL-4B (Sigma Chemical Co., U.S.A.) was pre-swollen in 0,1M phosphate buffer pH7. A 1 mL syringe fitted with a sintered glass frit was used as the column. This was attached to a peristaltic pump, UV-recorder and fraction collector.

200 µL of a CSF sample with an elevated peak between the spacers valine and leucine was added to the column previously washed with the phosphate buffer and the CSF was slowly eluted from the column. The fractions comprising the effluent peak were pooled and concentrated in B15 Minicon macroconcentrator (Amicon Corp., Danvers, U.S.A).

The concentrated sample (10 μL) with spacers was examined isotachophoretically in the MES/Ammediol leading electrolyte system.

6.4.2.2 Results

The isotachopherogram of the effluent from the column shows that the component between valine and leucine was bound to the column (Figure 6.11b), indicating that it is IgG. Other components in the immunoglobulin region are slightly reduced which is probably due to inadequate concentration.

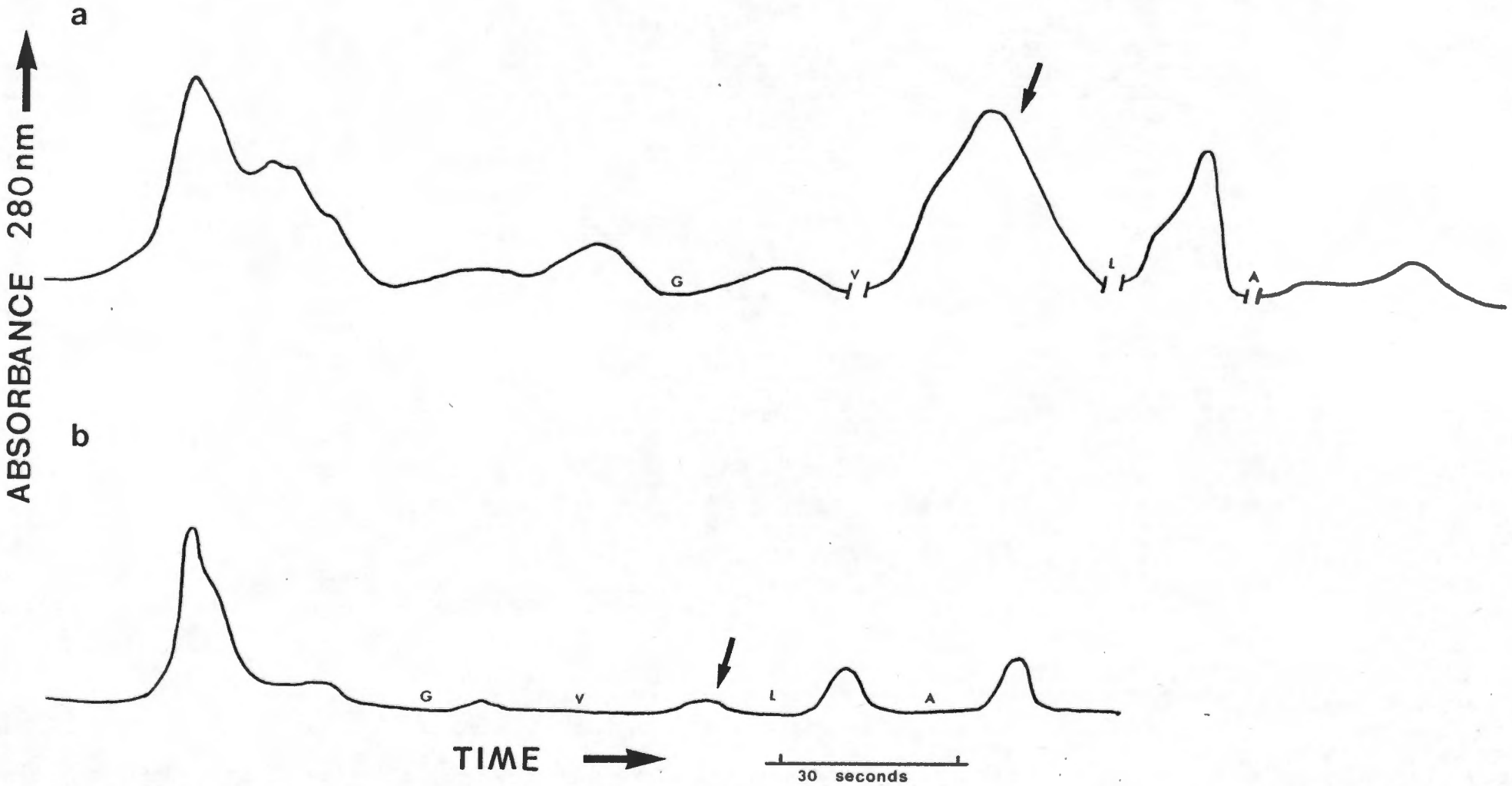
6.5 DISCUSSION

In this Chapter, a variety of different techniques were used in an attempt to identify some of the peaks in the CSF separation pattern. In some cases - e.g. doping experiments, a conclusive answer could not be given as the supposedly pure proteins used were, in fact, contaminated with other proteins. This shows the sensitivity of the isotachophoretic technique as, by other electrophoretic criteria, the protein acquired was given as 99% pure.

However, it is possible to get some indication as to where certain proteins appear in the CSF profile using doping experiments. Under the electrolyte conditions used in the present study, a number of the proteins added to the CSF, namely pre-

FIGURE 6.11

Isotachopherogram of a CSF with an increased component between the amino acid spacers valine and leucine (a) eluted through a protein A Sepharose column (b).


a
b
ABSORBANCE 280nm
G
V
L
A
G
V
L
A
TIME
30 seconds

albumin, orosomucoid, glycoprotein and α_1 antitrypsin, separate with the albumin and, thus, cannot be distinguished from each other. It would be necessary to change the electrolyte system, possibly to one with lower pH values, in order to separate these components. Haptoglobin, α_2 macroglobulin and transferrin appear in decreasing order of mobilities as UV absorbing peaks behind the albumin peak. The separations are still not ideal. Associated with the addition of α_2 macroglobulin to the CSF is a non-UV absorbing zone that migrates with the amino acid spacer glycine.

The IgA fraction in the CSF lies between the amino acid spacers, glycine and valine. This has also been noted by Delmotte (1977) in his examination of serum samples with IgA globulinanaemia.

The immunoglobulin fraction is artificially spaced into three UV-absorbing zones with the amino acids, valine, leucine and β alanine. These amino acids were added to aid in the interpretation of the CSF profile. The addition of IgG resulted in all three regions being increased, but the major increase was between the amino acid spacers, leucine and β alanine. A similar profile of increase occurs when both monoclonal or polyclonal IgM is added to the CSF. It would be anticipated that monoclonal IgM would occur as a single peak in the profile. However, this is not the case. Delmotte (1977) was unable to detect IgM in sera with high levels of IgM, which he

ascribes to aggregation at the low salt concentrations. It appears unlikely that in the present study, the increases seen in the immunoglobulin region after addition of IgM are due to contaminating IgG.

Investigations into the identity of the three UV-absorbing components of mobility greater than albumin that appear to be normal constituents of CSF, resulted in a number of observations. From the dialysis experiments, it was concluded that these FRP have molecular weights less than 1000 daltons. They are highly mobile, having mobilities greater than MES ion but less than the choride ion.

The addition of a variety of low molecular weight substances eventually led to the identification of two of the components in the front running region. The first peak was identified as folic acid.

It should be noted that although the first peak was identified as folic acid, the brain cannot utilize folic acid in this form and it is reduced to the methyl tetrahydrofolate form which is actively transported from the blood into the CSF via the choroid plexus and folic acid is transported out of the CSF (Spector and Lorenzo, 1975).

The CSF folate acid concentration is 15-60 ng/mL (Geigy Scientific Tables) and this is 2,5 to 5 times higher than in the

serum (Fishman, 1980). In the isotachophoretic analysis of serum, the presence of a component in the position of folic acid is not usually seen (unpublished observations). Although the significance of changes in the CSF folic acid concentration in neurological diseases is unknown, Isager (1970) reports that serum folate levels are significantly lower in multiple sclerosis patients, than those of a control group. Patients suffering from chronic neurological diseases - e.g. peripheral neuropathy, myelopathy and organic brain disease, also have increased serum folate deficiences.

The third FRP was found to be uric acid. The addition of uric acid resulted in a peak increase. A CSF sample with an elevated peak in the position of FRP3, treated with uricase, showed a reduction of this peak and the presence of allantoin, the breakdown product, which does not absorb at 280 nm. The position of the enzyme was also noted. This CSF sample was subsequently found to have a uric acid concentration 4 mg/dL which is above the normal concentration in the CSF of 0,25 mg/dL (Fishman, 1980).

Uric acid is a breakdown product of purine metabolism and the purines are the nitrogenous bases derived from the breakdown of nucleic acids. Elevated levels of CSF uric acid have been found in patients with cerebral atrophy (Farstad et al., 1965; Young and Crampton, 1974), alcohol withdrawal (Carlsson and Dencker, 1973) and with increasing age (Praetorius et al.,

1957) and in pre-eclamptic and eclamptic patients (Morrison et al., (1972). Elevated levels of uric acid in the serum may also be reflected in the CSF (Fishman, 1980).

A damaged blood-brain barrier may result in an increase in CSF uric acid and this is confirmed by isotachophoretic studies of CSF samples where there is an obvious increase in total protein the uric acid peak is also increased However, in a number of cases where the protein levels are normal, an increased uric acid peak may be found. These CSF samples are often from patients with degenerative brain disease, multiple sclerosis and GBS where lesions responsible for neuronal damage may result in increased levels of uric acid in the CSF.

Investigations to identify the elevated UV-absorbing component in viral meningitis, caused by a variety of viruses - e.g. mumps virus, enteroviruses and Coxsackie viruses, proved that this component was not IgM, one of the first immunoglobulins to appear during an infection.

Both the adsorption studies and treatment of a CSF sample with an IgM denaturing agent, did not remove or even reduce this component.

Beck (1981) has suggested that viral antibody activity resides exclusively in the IgG_3 subclass. As Protein A specifically binds human IgG_1, IgG_2 and IgG_4 subclasses (Kronvall and

Williams, 1969), it may be possible to determine the subclasses of the IgG represented by the component between valine and leucine. Using the protein A-Sepharose, the increased CSF component was not recovered in the effluent from the column, indicating that it is IgG but not the sub-class IgG_3. However, it may be one of the other three subclasses. It should be noted that the different IgG subclasses have been successfully separated by isotachophoresis (Hedlund et al., 1979).

The possibility also exists that this component is a viral protein. However, this appears unlikely as this increased component is not restricted to a single viral group that may have a common protein.

CHAPTER VII

ISOTACHOPHORETIC SEPARATION PROFILES OF CSF FROM CONTROL CASES AND PATIENTS WITH INFECTIOUS OR CHRONIC NEUROLOGICAL DISORDERS

7.1 INTRODUCTION

CSF protein abnormalities are associated with either blood-brain barrier damage with the resultant increase in cerebral capillary permeability, a selective elevation of immunoglobulins due to synthesis within the central nervous system (CNS) (Tibbling et al., 1977 and Schuller and Sagar, 1981) or serum protein abnormalities which are reflected in the CSF. These aberations may include prominent transferrin, tau and gamma-trace fractions and increased gamma globulins as seen when the CSF proteins are separated by electrophoresis (Siden and Kjellin, 1978). The presence of discrete bands of restricted homogenicity within the diffuse gamma region was first seen in 1960 (Lowenthal et al., 1960) and later described as an oligoclonal aspect (Laterre et al., 1970).

This oligoclonal banding is found in over 90% of multiple sclerosis (MS) CSF samples (Link and Muller, 1971; Thompson et al., 1979; Laurenzi et al., 1980; Chu et al., 1983). However, this is not specific of this disease and may be found in sub-

acute sclerosing panencephalitis (SSPE) (Vandvik and Norrby, 1973; Link et al., 1973a; Norrby and Vandvik, 1975; Siemes et al., 1977; Siemens et al., 1982; Mehta et al., 1982; Chu et al., 1983), neurosyphilis (Laterre et al., 1970; Vartdal et al., 1982), optic neuritis (Link et al., 1973b), Guillain-Barré syndrome (GBS) (Link, 1973b; Link, 1975; Link et al., 1979), aseptic meningitis (Link and Muller, 1971), progressive rubella panencephalitis (Wolinsky, 1976; Vandvik et al., 1978), Candida meningoencephalitis (Iwashita et al., 1978) and cerebrovascular disease (Rostrom and Link, 1981).

A variety of electrophoretic techniques have been used to detect these protein abnormalities. Electrophoresis in agar and agarose (Johnson et al., 1977; Laurenzi and Link, 1978; Olssen and Nilsson, 1979) has been used to demonstrate oligoclonal banding but, where improved resolution is required, a more sensitive method should be used. Discontinuous electrophoresis of unconcentrated CSF samples in polyacrylamide gels is sufficiently sensitive to resolve oligoclonal bands (Cunningham, 1964; Monseu and Cumings, 1965; Takeoka et al., 1976; Hochberg and Wolfson, 1979; Iivanainen et al., 1981). The superiority of isoelectric focusing over agarose electrophoresis for the separation of CSF proteins was first described in 1970 (Fossard et al., 1970). This method has since been used by a number of workers (Delmotte, 1971; Kjellin and Vesterberg, 1974; Stibler and Kjellin, 1976; Siden and

Kjellin, 1978; Stibler, 1978; Laurenzi and Link, 1978; Nilsson and Olsson, 1978; Olsson and Nilsson, 1979; Hosein and Johnson, 1981; Mattson et al., 1981; Kostulas and Link, 1982). These two electrophoretic methods, discontinuous polyacrylamide gel electrophoresis and isoelectric focusing, have so improved the resolution and separation capacity that much information may now be derived from the analysis of CSF proteins in neurological diseases of a chronic and/or infectious nature.

Isotachophoresis was initially only used in the examination of low molecular weight compounds (up to 3000 daltons) (Hjalmarsson and Baldesten, 1981), as stability of the separated zones in the capillary was a problem. With the addition of hydroxypropyl methylcellulose and other stabilizers to the leading electrolyte, the development of a high resolution UV detector and the spacer technique, the separation of proteins has become possible.

Arlinger and Routs (1970) were first in demonstrating the usefulness of isotachophoresis in the separation of proteins, namely haemoglobin and ceruloplasmin and the effect of ampholytes on the separation pattern.

The first application of isotachophoresis for the analysis of CSF proteins was by Kjellin et al. (1975a and 1975b). They compared normal CSF with CSF samples from patients with MS and

chronic meningoencephalitis and found abnormalities in the gammaglobulin region. Amino acids and ampholytes were used to obtain a better resolution.

The increased gammaglobulin levels in MS were confirmed in papers by Delmotte (1977), Kjellin (1976) and Kjellin and Siden (1978). Delmotte (1977) noted that the distinctive, and clearly visible, oligoclonal banding seen by isoelectric focusing could not be demonstrated when the CSF was examined by isotachophoresis.

The specific examination of the gammaglobulin region by cationic isotachophoresis (Kjellin and Hallander, 1979a; Kjellin and Siden, 1978) has shown differences between control cases and MS patients, especially in the low mobility gammaglobulin region. These results have been confirmed with anionic isotachophoresis in polyacrylamide gel tubes (Kjellin and Hallander, 1979b).

It is with this background of information that a study was initiated to examine other disease patterns, especially those of an infectious nature - e.g. viral and bacterial meningitis, as well as MS, SSPE and GBS.

7.2 MATERIALS AND METHODS

Anionic isotachophoresis was carried out as described in Chapter III, Materials and Methods. The spacer solutions of ampholytes and amino acids were used as previously described (Chapter III).

The CSF samples were obtained from the Virology and Bacteriology Diagnostic Laboratories of the Department of Medical Microbiology, University of Cape Town. After low speed centrifugation to remove cellular debris, the samples were stored at -20°C until required.

All CSF samples were examined without prior treatment - i.e. no concentration or dialysis procedures.

Twenty-two control CSF samples were obtained from patients subjected to lumbar puncture for non-inflammatory diagnostic procedures such as myelography for disc problems, back pain, leg pain and brachial plexus injury (Table 7.1).

The CSF total protein concentration was determined on these control samples by the Coomassie Brilliant Blue method (Johnson and Lott, 1978) and Lowry method (Lowry et al., 1951), and the albumin and IgG concentrations by radial immunodiffusion to establish the normal range (See Appendix B).

TABLE 7.1 - TABLE OF ALBUMIN, IgG AND TOTAL PROTEIN CONCENTRATIONS OF THE CONTROL CASES AND THE REASON FOR LUMBAR PUNCTURE

Sample	Total Protein Conc. (mg/dL)		Albumin Conc. (mg/dL)	IgG Conc. (mg/dL)	Diagnosis
	CBB*	Lowry			
CSF731	16,0	17,0	15,0	-	Cervical brachial plexus injury
CSF891	21,5	23,5	15,0	1,2	Brachial plexus injury
CSF623	31,0	30,5	12,0	0,8	Scoliosis
CSF929	37,5	48,5	23,0	2,6	Sudden onset of paraplegia
CSF897	13,5	20,5	12,0	-	Torticolis
CSF944	40,5	31,0	21,0	2,8	Sublimation L 4-5
CSF854	26,0	22,5	16,0	0,8	Lumbar disc
CSF825	48,0	50,0	23,0	0,8	Brachial plexus injury
CSF933	28,0	27,0	19,0	2,0	Disc lesion
CSF799	40,5	41,5	22,0	3,8	Spastic paraperesis
CSF634	70,5	63,0	25,0	2,8	Neck pain
CSF867	30,5	31,0	23,0	0,8	Sclerotenis disc
CSF943	13,0	9,0	12,0	0,8	Disc lesion
CSF595	23,5	18,0	17,0	-	Butterfly wedge vertebra
CSF515	25,0	27,5	20,0	-	Sacral neurofibroma
CSF559	65,5	61,5	25,0	0,8	Spondylitis
CSF598	28,5	26,5	23,0	-	Low back pain
CSF642	26,0	23,5	21,0	-	Disc problem
CSF616	40,0	43,0	19,0	-	Low back and leg pain
CSF571	61,5	64,5	25,0	-	Recurrent disc problem
JK	27,0	20,0	26,0	3,5	Lumbar disc
UD	37,0	47,5	25,0	2,8	Lumbar disc
X	34,0	33,7	19,5	1,87	
SD	±15,8	±16,3	± 4,6	±1,13	

* Coomassie Brilliant Blue Method
X Mean value
SD Standard deviation

The CSF was categorised as viral meningitis when the virus was cultured from the sample. A total of 104 CSF samples were examined by isotachophoresis. A variety of virus species were isolated and included enteric cytopathogenic human orphan virus type 4 (ECHO 4) (53); Coxsackie A and B (18); mumps virus (14) and 19 non-polio-non Coxsackie enteroviruses.

Twelve bacterial meningitis CSF samples caused by a range of organisms: Diplococcus penumoniae (3); Neisseria meningitidis (6), Group A Streptococcus (1); a Proteus species and 1 Clostridium welchii, were examined.

Three proven cryptococcal meningitis (Cryptococcus neoformans) CSF samples were also examined.

Twenty Guillain-Barré Syndrome CSF samples were obtained, as were 33 confirmed or probable MS samples. In four cases, more than one CSF sample was available. Some of these samples had been taken within two days and, in one case, D.D., three CSF samples taken in 1977, 1978 and 1980 were available for isotachophoretic analysis.

An extensive investigation of the SSPE cases referred to Groote Schuur Hospital was undertaken. Thirty-seven cases of SSPE diagnosed between 1971 and 1984 with 42 CSF samples were

examined. In five cases, more than one CSF sample was available. All the cases had measles antibody in the CSF with titres as determined by the complement fixation method of 1:16 - 1:1280.

7.3 RESULTS

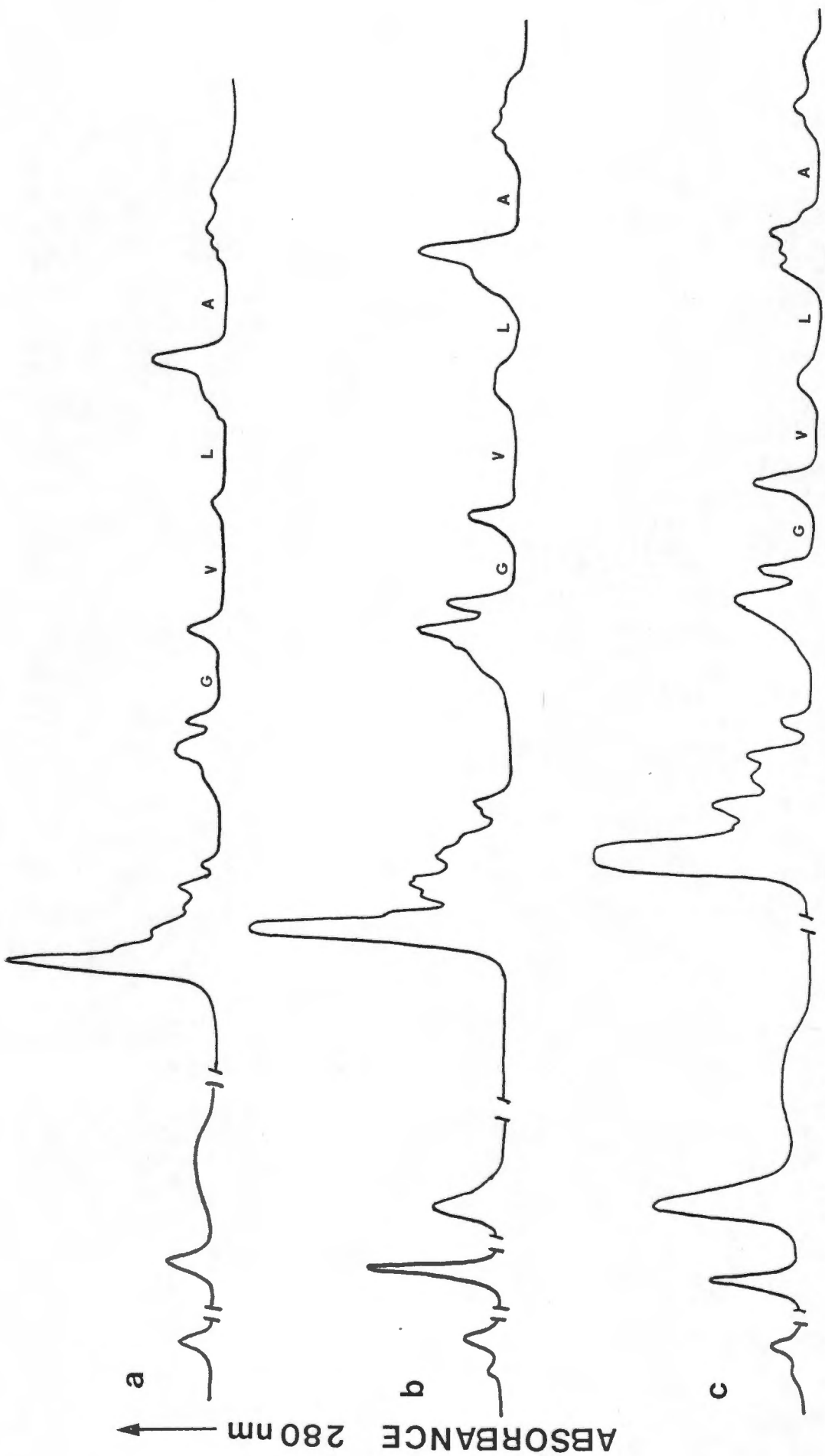
7.3.1 Isotachopherograms of CSF from control cases

The average CSF albumin and IgG concentrations determined by the RID method for the 22 control cases was 19,5 mg/dL and 1,87 mg/dL respectively. Differences in the total protein concentration, as determined by the Coomassie Brilliant Blue and Lowry methods, occur in individual CSF samples (Table 7.1). However, a mean of 34,0 mg/dL and 33,7 mg/dL for the two methods was found respectively. These results are comparable with published data (See Appendix B).

Examples of the isotachophoretic patterns obtained from the control subjects are given in Figure 7.1. The front running peaks are always present and may vary in size and shape. The peak between the amino acid spacers, leucine and β alanine, is dominant in the immunoglobulin.

FIGURE 7.1

Isotachopherograms of three normal CSF samples.


a
b
c
G
V
L
A
ABSORBANCE 280 nm
TIME
30 seconds

The peak areas were recorded and these results, expressed as a percentage of the total area, are given in Table 7.2. The mean albumin and IgG concentrations are 28,5% ± 7,5 and 17,7% ± 5,1 respectively.

The three immunoglobulin peaks expressed as a percentage of the total IgG confirms that the peak between the spacers leucine and β alanine is the dominant peak - 62,3% ± 15,3.

The mean globulin to albumin ration (IgG/Alb) is 0,675 ± 0,264.

7.3.2 Isotachopherograms of CSF from viral meningitis cases

The isotachophoretic profiles obtained from this group show two main patterns. The majority (60,5%) of the CSF samples examined had a normal CSF profile (Figure 7.2a). However, in approximately 39,5% of the cases, an increased peak was found between the amino acid spacers valine and leucine (Figure 7.2b, c). The identity of this peak is unknown (See Chapter VI). This increase was not confined to a single virus genus which may indicate a specific viral protein. It occurs in the CSF from which unrelated viruses - e.g. Enterovirus and Paramyxovirus, were isolated. The albumin and other peaks follow the normal CSF profile. However, in some of the cases, the FRP may be slightly increased.

TABLE 7.2 - ISOTACHOPHORETIC RESULTS OF NORMAL CSF SAMPLES

Sample	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
			Val-Leu	Leu-Ala	Ala	
CSF731	24,3	16,3	35,8	50,4	13,7	0,672
CSF891	21,8	20,6	7,5	82,2	10,2	0,945
CSF623	34,0	18,8	16,6	78,7	4,5	0,552
CSF929	27,2	17,9	13,5	75,6	10,8	0,658
CSF897	23,8	23,4	7,1	67,1	25,6	0,982
CSF944	13,3	13,8	19,8	58,7	21,4	1,065
CSF854	24,7	16,7	7,9	70,6	21,4	0,675
CSF825	30,3	10,8	27,5	36,5	35,8	0,356
CSF933	23,0	11,7	14,9	78,5	6,5	0,509
CSF799	23,0	12,9	25,2	56,6	18,0	0,561
CSF634	31,9	13,7	11,8	67,1	21,0	0,428
CSF867	24,2	16,1	14,4	69,9	15,6	0,664
CSF943	23,4	11,7	28,2	24,3	47,4	0,501
CSF595	-	16,9	-	53,1	46,8	-
CSF515	23,1	15,9	14,6	50,2	31,7	0,688
CSF559	39,8	20,7	8,7	83,8	7,3	0,519
CSF598	44,2	16,4	-	72,3	27,6	0,371
CSF642	35,5	29,1	29,0	51,6	19,2	0,818
CSF616	40,4	18,3	-	67,2	32,7	0,453
JK	31,5	20,5	18,5	49,2	32,1	0,650
UD	21,3	30,4	20,0	64,9	34,5	1,430
X	28,5	17,7	17,8	62,3	23,0	0,674
SD	7,5	5,1	8,4	15,3	12,4	±0,264

X Mean value
SD Standard Deviation
TP Total protein

NOTE: The percentages of proteins calculated from the isotachophorerogram are not the absolute values. This is because:

(a) the albumin peak is not pure, and

(b) the proteins are migrating in ampholytes which tend to expand the curves.

These percentages are, however, useful in comparing different isotachopherograms.

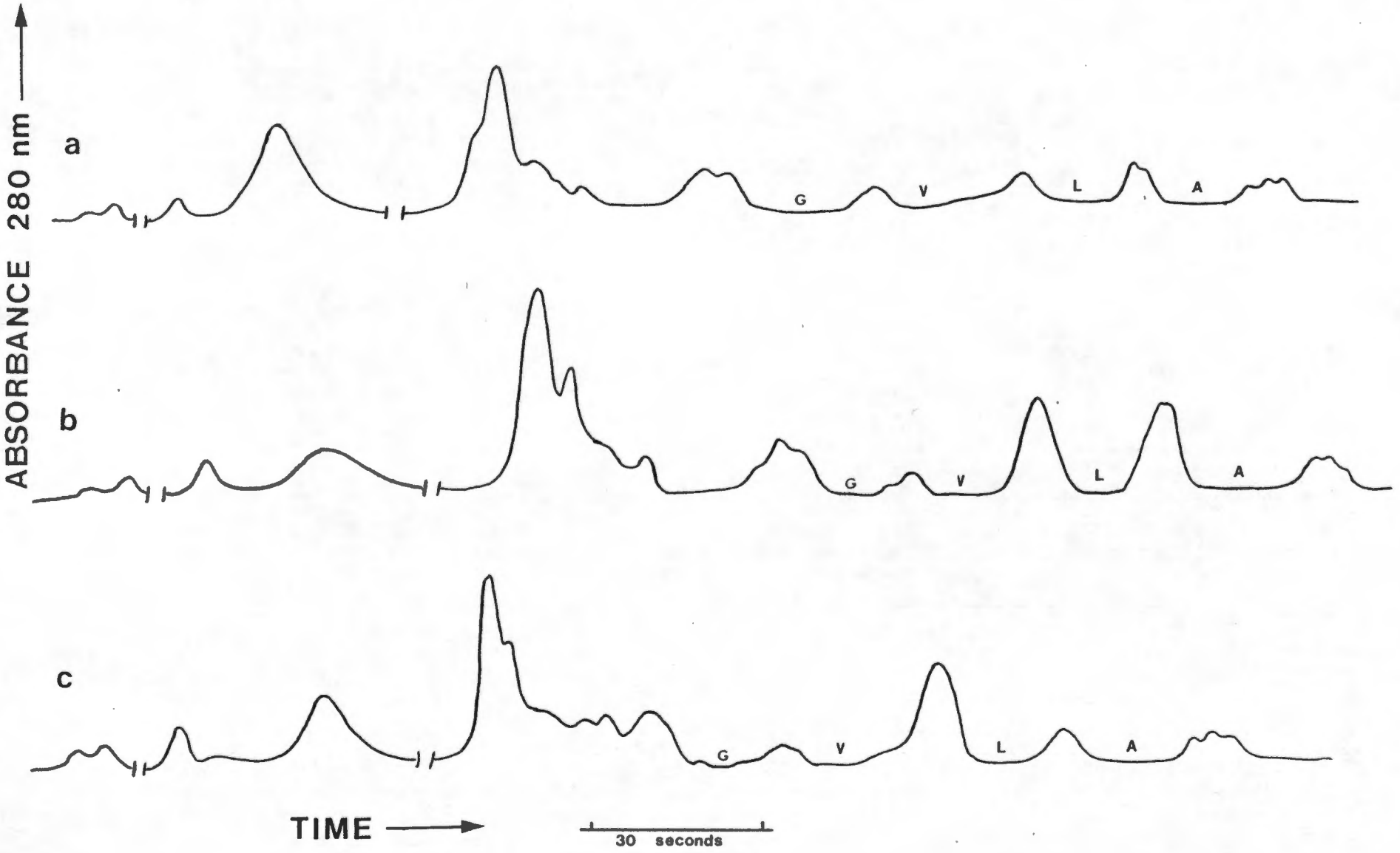
FIGURE 7.2

Isotachopherograms of viral meningitis CSF samples

(a) mumps meningitis with a protein separation profile similar to normal

(b) non-polio non-Coxsackie enterovirus isolated from CSF showing an increased peak between valine and leucine

(c) Coxsackie B_5 isolated from CSF showing an increased peak between valine and leucine


ABSORBANCE 280 nm
a
b
c
G
V
L
A
TIME
30 seconds

The area of the peaks was recorded and these results were expressed as a percentage of the total area (Table 7.3). The albumin and IgG levels for the normal CSF profiles is 29,3% ± 8,4 and 20,9% ± 7,8 respectively, and the IgG/Alb ratio is 0,802 ± 0,443. For the CSF profiles with the elevated peak, the albumin and IgG concentrations are 25,9% ± 6,4 and 27,2% ± 8,6 respectively. The IgG/Alb ratio is 1,077 ± 0,480.

On examination of the distribution of the three IgG fractions expressed as a percentage of the total IgG area, 27 IgG peaks between valine and leucine have increased areas above 35% of total IgG area (Table 7.3). This represents 25,9% of the total number of specimens examined. However, it is also essential to look at these values in conjunction with the isotachophoretic patterns. Of the 104 specimens, 41 specimens show an elevated peak. This discrepancy is in part due to the entire peak area not being recorded. Thus, the peaks must be qualitatively compared in height and area with other IgG peaks.

7.3.3 Isotachopherograms of CSF from bacterial meningitis

The isotachopherograms of confirmed bacterial meningitis contrast markedly with the pattern obtained from viral meningitis (Figure 7.3). The peaks are increased in all cases, but the degree of increase is varied. The FRP may also be elevated. An increase in the peak betwen valine and leucine is seen in only some of the samples examined, and this increase is not as marked as that in viral meningitis.

TABLE 7.3 - ISOTACHOPHORETIC RESULTS OF 104 VIRAL MENINGITIS CSF SAMPLES

Sample	Virus	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
				Val-Leu	Leu-Ala	Ala	
1	Echo 4	26	28	8	79	13	1,101
2	Echo 4	29	23	11	74	15	0,791
3	Echo 4	28	24	12	66	22	0,862
4	Echo 4	33	13	10	70	20	0,385
5	Echo 4	34	24	48*	45	7	0,691
6	Echo 4	19	44	50*	46	4	2,343
7	Echo 4	19	16	12	68	20	0,860
8	Echo 4	15	21	11	68	21	1,406
9	Echo 4	31	15	8	41	51	0,483
10	Echo 4	35	16	18	41	41	0,443
11	Echo 4	-	38	21*	58	21	-
12	Echo 4	-	42	19*	55	26	-
13	Echo 4	-	36	36*	36	28	-
14	Echo 4	47	27	14	-	-	0,568
15	Echo 4	18	32	40*	39	21	1,772
16	Echo 4	33	23	4	70	26	0,710
17	Echo 4	28	26	39*	47	14	0,954
18	Echo 4	33	22	35*	33	32	0,658
19	Echo 4	20	24	3	78	19	1,190
20	Echo 4	34	15	10	44	46	0,444
21	Echo 4	32	13	25	61	14	0,404
22	Echo 4	35	20	73*	23	4	0,557
23	Echo 4	25	20	73*	20	7	0,805
24	Echo 4	35	7	52*	37	11	0,205
25	Echo 4	27	9	-	80	20	0,305
26	Echo 4	31	15	74*	25	1	0,490
27	Echo 4	30	9	21	45	34	0,311
28	Echo 4	21	21	24	70	6	0,975
29	Echo 4	25	22	6	74	20	0,872
30	Echo 4	33	29	36*	59	5	0,879
31	Echo 4	24	24	24	55	21	1,021
32	Echo 4	38	20	4	82	14	0,514
33	Echo 4	39	21	9	57	34	0,534
34	Echo 4	25	31	67*	27	6	1,230
35	Echo 4	32	28	32	54	14	0,447
36	Echo 4	34	22	43*	46	11	0,653
37	Echo 4	26	25	6	81	13	0,929
38	Echo 4	25	27	16	61	23	1,069
39	Echo 4	33	24	4	81	15	0,717
40	Echo 4	18	26	2	87	11	1,456
41	Echo 4	27	31	27*	57	16	1,143
42	Echo 4	46	27	3	88	9	0,584

Sample	Virus	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
				Val-Leu	Leu-Ala	Ala	
43	Echo 4	34	27	42*	54	4	0,778
44	Echo 4	25	27	5	74	21	1,069
45	Echo 4	29	22	8	84	8	0,744
46	Echo 4	38	23	3	79	18	0,611
47	Echo 4	26	11	-	83	17	0,413
48	Echo 4	28	31	51*	45	4	1,111
49	Echo 4	32	31	59*	26	15	0,971
50	Echo 4	48	16	27	59	14	0,338
51	Echo 4	27	16	7	84	9	0,604
52	Echo 4	29	28	53*	44	3	0,941
53	Echo 4	28	22	36	57	4	0,781
54	Mumps	27	20	15*	75	10	0,748
55	Mumps	28	7	26	26	48	0,268
56	Mumps	23	21	21	60	16	0,828
57	Mumps	28	22	62*	27	11	0,899
58	Mumps	27	7	-	40	60	0,265
59	Mumps	24	26	70*	18	12	1,110
60	Mumps	49	13	28	32	40	0,269
61	Mumps	22	26	54*	27	19	1,187
62	Mumps	19	23	31*	50	19	1,184
63	Mumps	30	26	19	69	12	0,863
64	Mumps	24	21	54*	39	7	0,889
65	Mumps	29	28	20*	68	12	0,957
66	Mumps	28	17	24	54	24	0,620
67	Mumps	13	42	27	64	9	3,184
68	Cox B3	22	19	34*	51	15	0,836
69	Cox B3	16	33	29*	59	12	2,031
70	Cox B3	26	23	10	65	25	0,887
71	Cox B3	23	24	69*	22	9	1,046
72	Cox B3	17	20	14	53	33	1,160
73	Cox B3	24	13	12	67	21	0,533
74	Cox B3	14	8	34*	56	10	0,601
75	Cox B3	28	25	6	75	19	0,898
76	Cox B3	20	29	87*	5	8	1,407
77	Cox B3	30	42	21*	68	11	1,411
78	Cox B3	24	28	32*	55	13	1,197
79	Cox B3	32	19	18*	60	22	0,561
80	Cox B5	18	30	63*	23	14	1,657
81	Cox B5	29	14	26	57	17	0,478
82	Cox B5	20	28	30	53	17	1,445
83	Cox A	29	17	9	72	19	0,599
84	Cox A9	38	62	-	75	25	1,655
85	Cox A	15	23	68*	19	13	1,521
86	NPNC	29	26	11	70	19	0,922
87	NPNC	24	15	14	67	19	0,624
88	NPNC	25	21	18	66	16	0,842

Sample	Virus	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
				Val-Leu	Leu-Ala	Ala	
89	NPNC	23	24	8	79	13	1,029
90	NPNC	24	15	-	69	31	0,634
91	NPNC	26	25	13	75	12	0,983
92	NPNC	16	12	-	57	43	0,774
93	NPNC	18	38	26*	68	6	2,084
94	NPNC	33	24	60*	21	19	0,717
95	NPNC	48	40	10	55	35	0,827
96	NPNC	30	27	-	58	42	0,882
97	NPNC	32	48	33*	38	29	1,493
98	NPNC	48	44	25	47	28	0,919
99	NPNC	40	37	24	55	21	0,922
100	NPNC	37	13	19	52	29	0,363
101	NPNC	18	26	21	55	24	1,405
102	NPNC	17	12	14	46	40	0,715
103	NPNC	38	15	11	46	43	0,394
104	NPNC	20	28	42*	44	14	1,390
NX±SD		29,3 ±8,4	20,9 ±7,8				0,802 ±0,443
X*±SD		25,9 ±6,4	27,2 ±8,6				1,077 ±0,480

* CSF samples with elevated peak between amino acid spacers valine and leucine.

NX mean value for CSF samples with normal protein profile.

X* mean value for CSF samples with elevated peak between amino acid spacers valine and leucine.

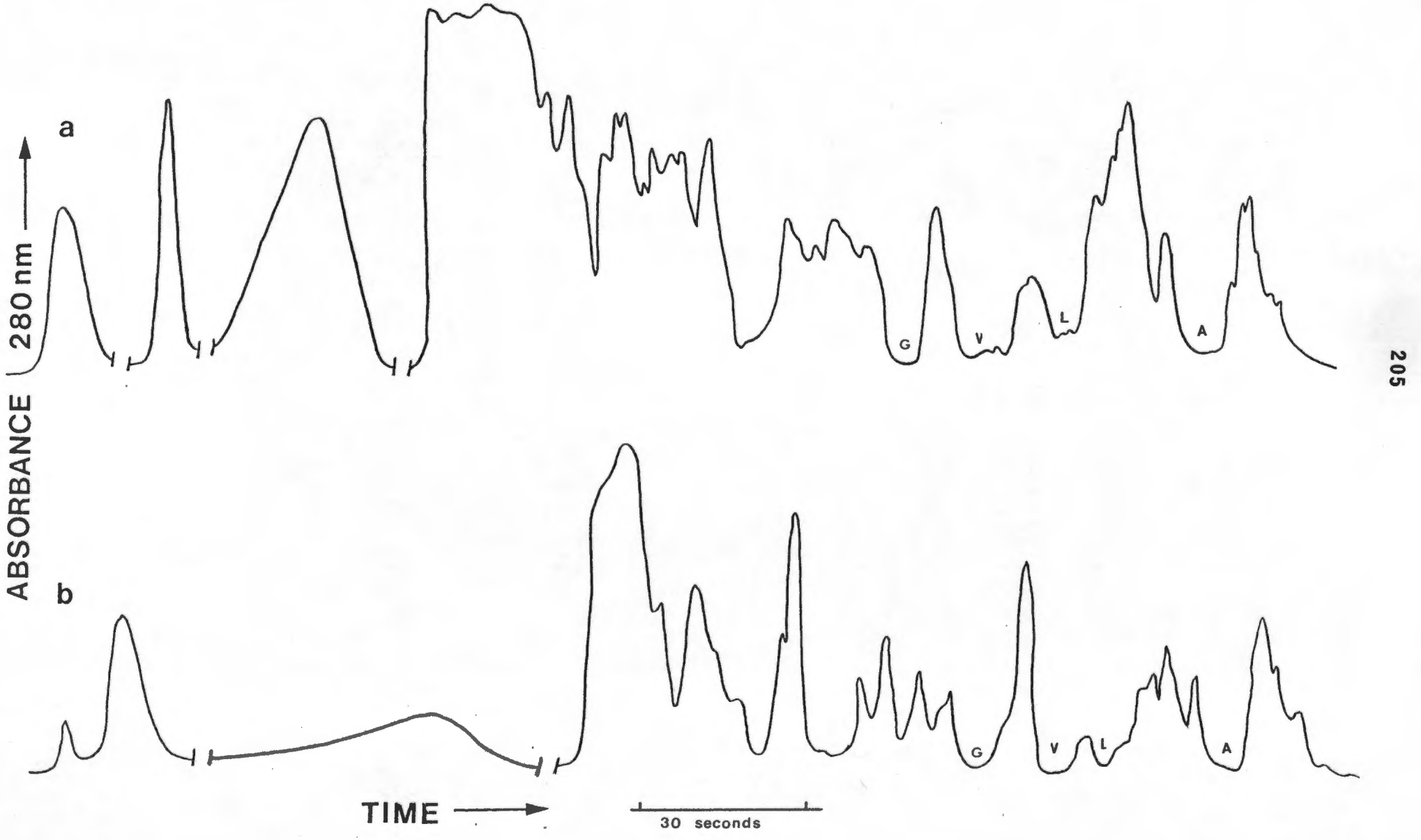
SD Standard Deviation

TP Total protein

NPNC non-Polio non-Coxsackie enterovirus

FIGURE 7.3

Isotachopherograms of bacterial meningitis CSF samples (a) Diplococcus pneumoniae, (b) Neisseria meningitides.


a
b
ABSORBANCE
280 nm
G
V
L
A
TIME
30 seconds

There does not appear to be a distinctive pattern for the different causal agents of the meningitis.

As the peak areas were too large to be accurately recorded, analyses on these samples were not performed.

7.3.4 Isotachopherograms of CSF from cryptococcal meningitis

In all three cases of meningitis caused by Cryptococcus neoformans a large UV-absorbing component was found with a mobility less than the slowest moving gammaglobulin fraction (Figure 7.4). The identity of this peak is unknown but could be a yeast protein. The other protein peaks in the separation profile, including the FRP, are increased.

7.3.5 Isotachopherograms of CSF from multiple sclerosis

The majority of the 32 MS cases show an overall isotachophoretic protein profile similar to that of the normal profile. However, there are a number of important differences. The FRP may be increased; albumin levels may be slightly raised and, most importantly, there is an enlarged immunoglobulin peak that occurs between the amino acid spacers leucine and β alanine (Figure 7.5).

In one case, D.D., three CSF samples taken over a period of years, 1977, 1978 and 1980, show the change in the CSF protein profile (Figure 7.6). The albumin peak is enlarged and

FIGURE 7.4

Isotachopherograms of two cryptococcal meningitis CSF samples.

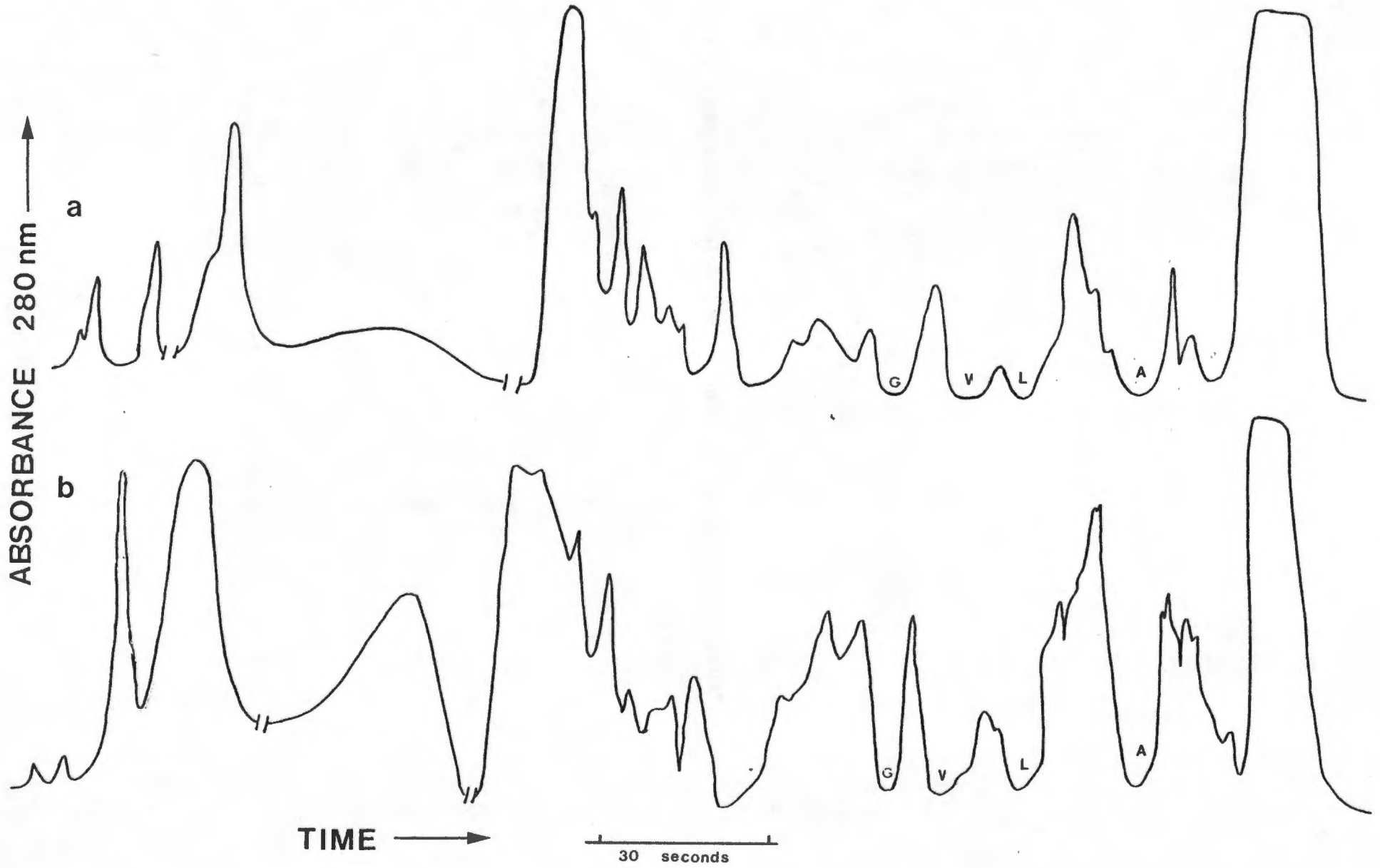

ABSORBANCE 280 nm
a
b
G
V
L
A
TIME
30 seconds

FIGURE 7.5

Isotachopherograms of three multiple sclerosis CSF samples.

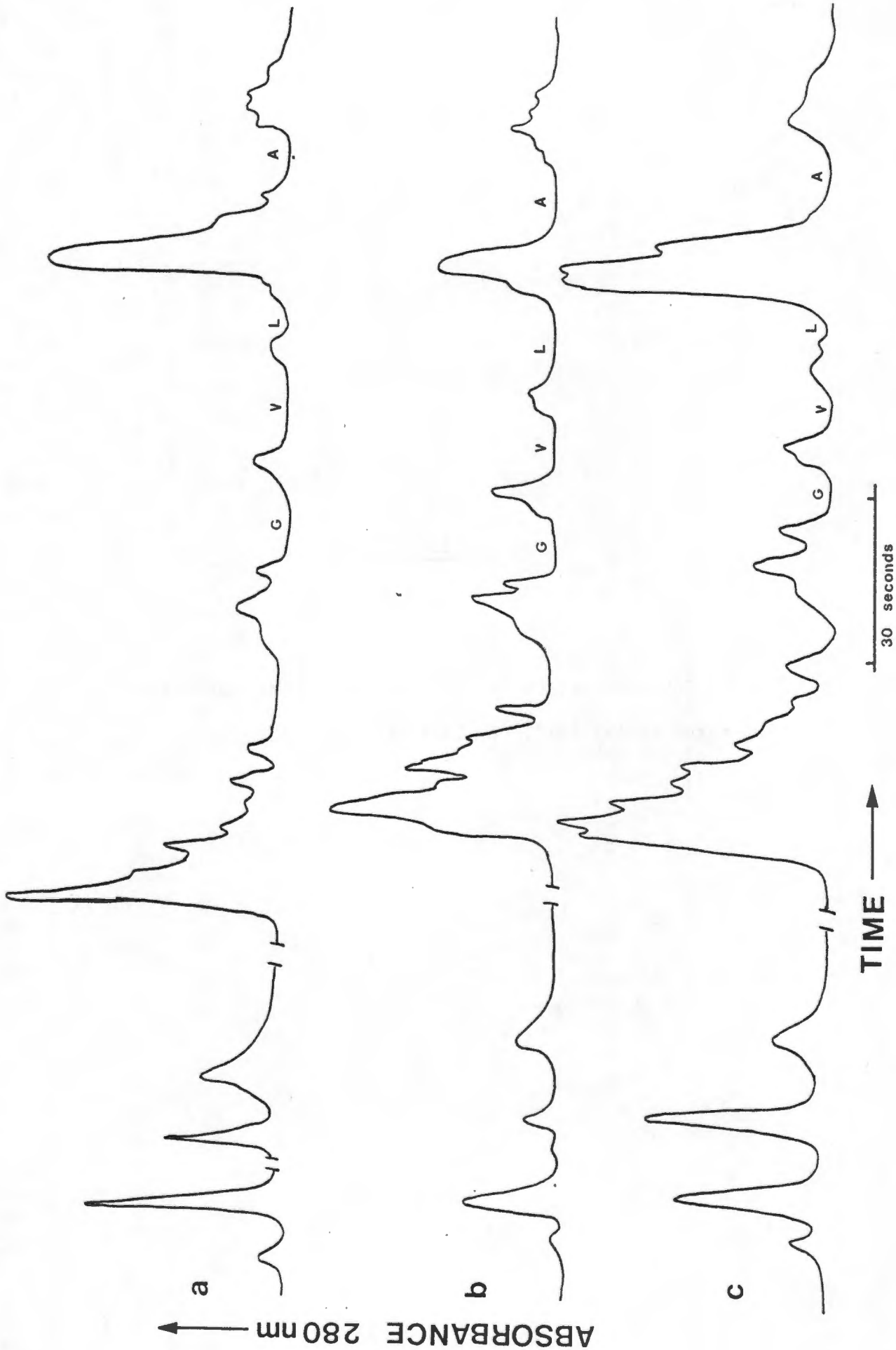
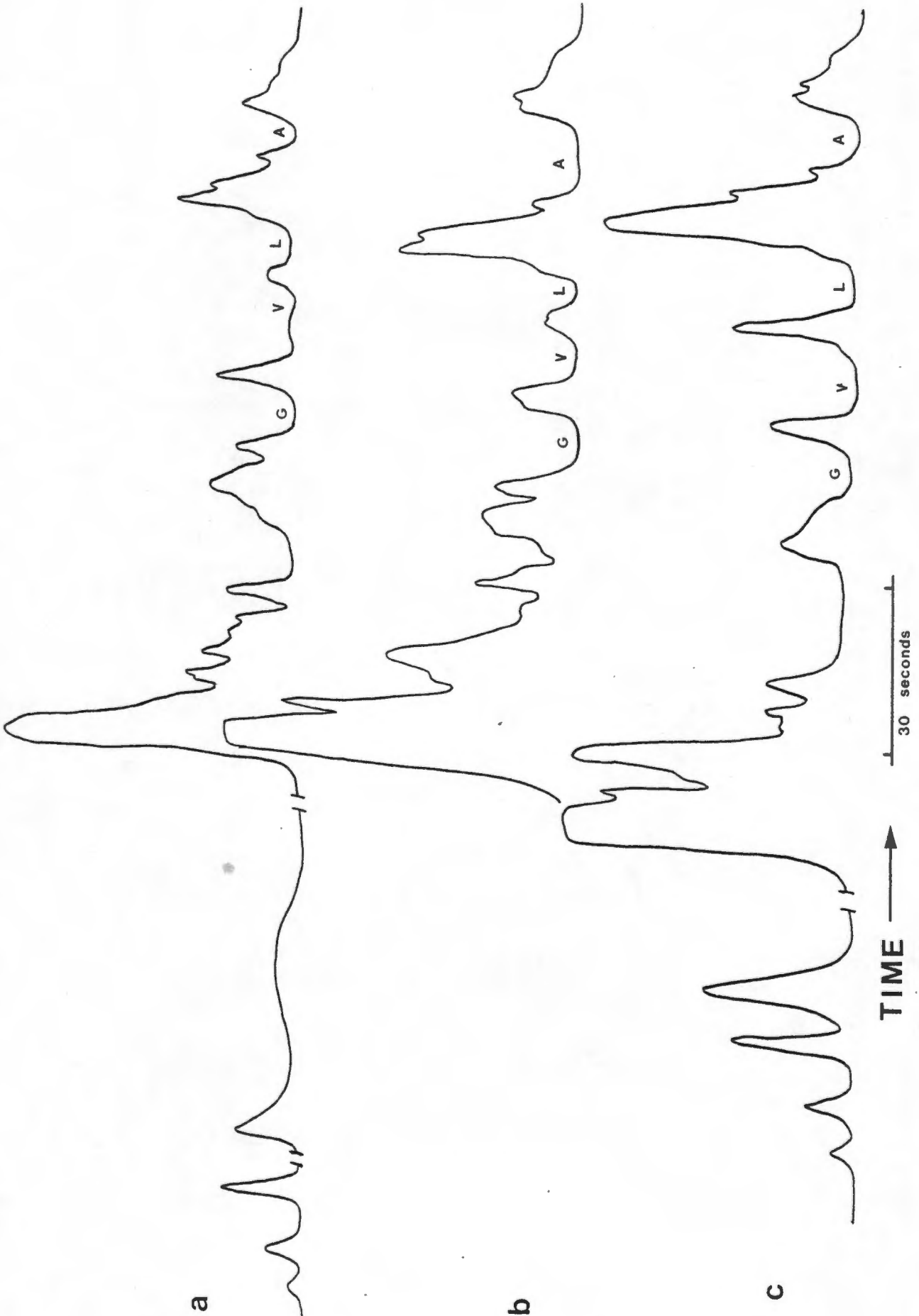

a
b
c
A
L
V
G
ABSORBANCE 280nm
TIME
30 seconds

FIGURE 7.6

Isotachopherograms of the CSF of an MS patient (D.D.) taken in (a) 1977, (b) 1978 and (c) 1980.


ABSORBANCE 280 nm
a
b
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G
V
L
A
TIME
30 seconds

remained at this level in all three specimens. The immunoglobulin peak between the spacers leucine and β alanine gradually increased over the same period.

Table 7.4 shows the isotachophoretic results of albumin and IgG expressed as a percentage of the total area. The IgG/Alb ratio is 1,045 ± 0,477 which is greater than the normal IgG/Alb ratio of 0,674.

7.3.6 Isotachopherograms of CSF from Guillain-Barré Syndrome

The isotachophoretic profiles of the 20 GBS CSF samples examined, vary greatly (Figure 7.7). In some cases an elevated albumin peak was found without the other protein components being elevated. In other cases a normal CSF profile was found. In all cases there was no distinctive increase in the immunoglobulin region. The FRP may also be increased.

The table of isotachophoretic results confirm that no elevated globulin levels are present (16,4 ± 5,3%) (Table 7.5).

7.3.7 Isotachopherograms of CSF from subacute sclerosing panencephalitis

Of the 37 SSPE cases examined by isotachophoresis, all but four showed a distinctive pattern (Figure 7.8). The immunoglobulins of mobility less than the amino acid spacer leucine were increased and especially those with a mobility

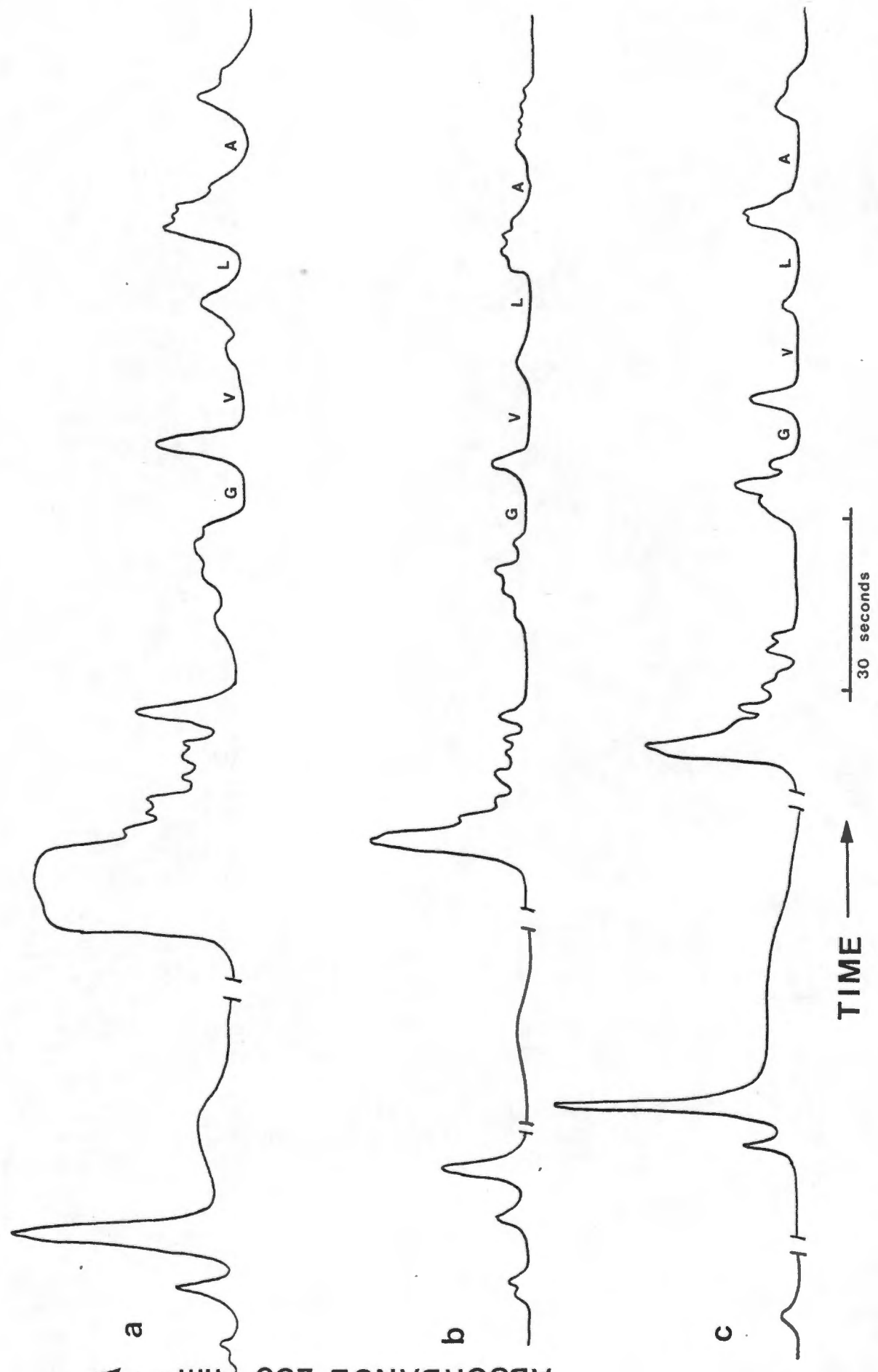
TABLE 7.4 - ISOTACHOPHORETIC RESULTS OF MULTIPLE SCLEROSIS CSF SAMPLES

Sample		Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
				Val-Leu	Leu-Ala	Ala	
KA	V78/927	29,5	15,0	6,6	73,5	19,8	0,511
LEW	V76/2474	26,2	24,0	16,5	5,3	30,4	0,916
	V76/2878	21,0	12,1	8,9	55,1	35,5	0,578
BU	V78/117	18,0	14,7	13,9	75,0	11,0	0,824
RO	V79/3017	21,3	26,3	8,8	68,4	22,6	1,236
	CSF907	24,2	26,4	12,3	49,2	38,4	1,091
DA	V77/1413	22,1	19,5	14,4	70,4	15,0	0,882
	V78/934	28,9	15,9	8,2	64,9	26,0	0,551
	V80/1049	28,1	23,3	16,1	67,6	16,2	0,829
CHA	V77/1368	13,8	15,1	11,6	81,7	6,5	1,092
OL	V78/2824	18,2	29,6	5,3	89,0	5,5	1,619
HE	V78/1889	28,0	13,7	17,8	53,3	28,8	0,491
	V78/1920	22,1	22,3	6,3	72,0	21,5	1,012
KAH	V76/975	21,3	25,0	34,3	51,5	14,0	1,173
OP	V78/3366	24,3	21,6	5,6	7118	22,5	0,888
ME	V78/770	30,5	24,0	6,4	71,8	13,7	0,789
VO	V77/1987	18,8	25,2	9,6	79,4	10,8	1,337
KE	V78/2217	24,1	21,0	10,2	76,6	13,1	0,870
WO	CSF906	24,8	15,4	14,5	70,7	14,6	0,623
PI	V78/256	20,3	22,9	9,4	71,6	18,8	1,129
VV	V79/1021	19,6	36,3	8,3	63,7	27,9	1,847
MO	V79/2841	21,4	39,9	7,0	71,1	21,8	1,860
VW	V77/1220	27,2	25,9	14,1	57,8	28,0	0,954
EN	V79/2394	32,2	18,1	8,9	71,9	19,1	0,562
TA	V78/441	26,6	22,8	8,5	49,9	41,5	0,858
RA	V76/2175	21,9	32,0	4,2	85,1	10,5	1,459
AN	V76/1991	20,0	29,0	4,3	79,7	15,8	1,445
BA	V78/670	25,9	13,2	-	79,5	20,4	0,509
BU	V78/1177	19,9	26,3	3,0	74,6	22,2	1,323
PE	V78/2606	22,2	12,6	13,9	66,8	19,2	0,568
JA	CSF905	19,8	24,0	10,8	58,5	30,5	1,215
JAC	V77/1766	10,9	30,1	5,3	86,9	7,7	2,743
GO	V77/3262	19,1	15,9	17,1	67,5	15,2	0,835
OK	V77/3262	27,9	12,7	14,6	66,1	19,1	0,458
PE	V76/2105	18,5	24,5	7,2	72,8	19,8	1,322
LE	V77/3341	10,1	15,6	7,4	67,9	10,2	1,543
CO	V78/1716	24,0	17,9	11,2	70,6	18,4	0,745
X		22,5	21,8	10,56	69,3	19,67	1,045
SD		±5,01	±6,82	±5,6	±10,3	±8,4	±0,477

X Mean value
SD Standard Deviation
TP Total protein

FIGURE 7.7

Isotachopherograms of three Guillain-Barré syndrome CSF samples.


ABSORBANCE 280 nm
a
b
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G
V
L
A
TIME
30 seconds

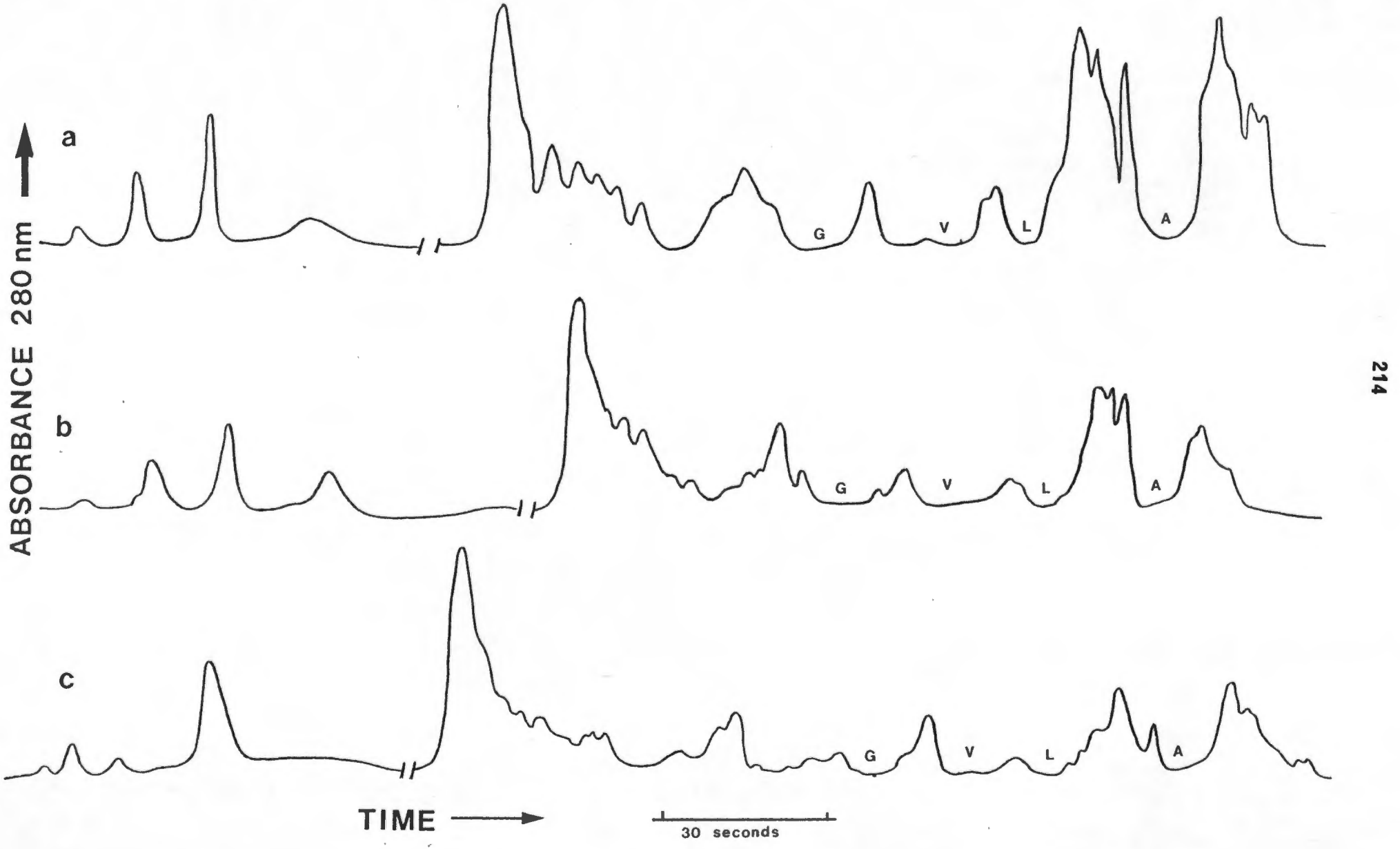
TABLE 7.5 - ISOTACHOPHORETIC RESULTS OF GUILLAIN-BARRÉ SYNDROME CSF SAMPLES

Sample	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
			Val-Leu	Leu-Ala	Ala	
MO	43,0	14,0	10,5	65,9	24,0	0,413
MU	24,3	17,2	26,9	50,8	22,1	0,700
HE	16,6	11,8	3,5	71,1	25,3	0,711
MA	29,4	26,6	7,0	73,0	21,2	0,900
VI	20,2	17,4	10,5	77,3	12,2	1,050
MA	11,6	8,0	17,4	68,8	13,7	1,150
FR	30,9	11,7	8,0	47,7	32,2	0,378
KO	21,6	9,2	15,0	36,1	48,7	0,428
DK	39,6	25,2	10,4	63,5	25,9	0,600
ER	33,0	14,1	3,4	82,9	13,3	0,420
DU	21,0	16,7	8,2	62,1	29,1	0,797
CU	34,3	14,3	5,0	70,4	24,4	0,424
CA	19,4	18,8	18,2	46,7	34,7	0,940
MO	-	18,9	16,1	60,5	23,3	-
BU	29,4	22,2	7,0	73,0	21,2	0,906
DY	32,4	19,1	9,3	67,5	23,0	0,591
JO	23,0	12,9	16,2	55,6	27,7	0,570
HA	27,0	11,4	10,9	71,1	17,9	0,421
CU	20,2	15,3	6,1	89,5	3,8	0,758
BE	17,6	25,0	4,9	43,5	51,6	1,440
X	25,3	16,4	10,7	63,4	24,7	0,723
SD	7,7	5,3	6,1	13,9	11,3	0,300

X Mean value
SD Standard Deviation
TP Total protein

FIGURE 7.8

Isotachopherograms of three SSPE CSF samples.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ABSORBANCE 280 nm
TIME
30 seconds

slower than β alanine. In the four atypical cases, the immunoglobulin fraction in the region slower than alanine was not increased. However, the peak between leucine and β alanine was elevated. In all cases, the albumin fraction remained within normal limits.

The distinctive oligoclonal banding so characteristic of SSPE, as seen by isoelectric focusing, was not present in these patterns. These restricted fractions would probably appear as sharp discrete peaks in the immunoglobulin region.

Table 7.6 gives the isotachophoretic results of albumin and IgG expressed as a percentage of the total protein and the three sub-fractions of the immunoglobulins expressed as a percentage of the total IgG. The IgG/Alb ratio of 1,831 ± 0,789 is above the normal ratio of 0,674 and indicates synthesis of immunoglobulins within the central nervous system.

TABLE 7.6 - ISOTACHOPHORETIC RESULTS OF SSPE CSF SAMPLES

Sample	Alb. % TP	IgG % TP	IgG Distribution %			IgG/Alb Ratio
			Val-Leu	Leu-Ala	Ala	
795 L K	10,6	36,9	6,7	45,9	47,2	3,455
793 M W	18,8	41,6	4,8	79,6	15,4	2,602
104 U D	12,4	26,9	4,1	54,6	41,2	2,190
	14,6	29,2	5,5	43,3	51,0	2,047
784 C C	22,3	40,0	8,3	33,5	58,1	1,787
93 W N	23,1	33,0	11,7	60,5	27,6	1,427
106 B W	11,1	24,4	6,9	49,2	43,9	3,187
97 L L	28,3	20,8	17,0	68,5	14,3	0,735
111 G T	28,5	26,0	12,7	56,9	30,3	0,909
70 S R	11,7	36,1	12,1	71,4	16,3	3,074
69 A P	16,3	28,6	13,3	57,0	28,6	1,754
72 A H	20,1	33,1	12,1	48,8	38,7	1,608
98 DP	21,8	28,6	5,0	51,5	43,3	1,314
787 M M	17,2	28,0	13,2	90,0	8,6	1,975
815 B N	31,0	39,2	5,9	38,5	55,4	1,261
802 M P	17,1	36,1	6,4	45,0	48,4	2,112
105 J V R	22,4	33,4	5,6	49,7	44,5	1,452
836 S S	22,3	22,7	7,7	26,1	66,1	1,010
837 H Q	10,2	35,6	-	47,2	52,7	3,468
	22,7	37,3	5,4	46,8	47,7	1,640
	25,5	38,0	5,2	64,0	30,6	1,489
823 M M	27,9	30,3	10,5	29,4	59,9	1,087
824 P K	19,7	37,2	3,8	47,5	48,5	1,883
831 M P	32,3	29,0	1,3	50,1	48,5	0,898
834 C P	24,8	38,2	4,0	53,0	42,9	1,535
	22,0	43,3	1,4	38,7	59,2	1,900
843 J L	-	32,1	9,8	43,4	46,6	-
842 M M	34,3	29,3	3,8	40,6	55,4	0,855
G	23,6	65,2	1,9	41,6	56,4	2,752
825 R B	28,5	32,3	14,8	61,0	25,0	1,136
812 C T	15,0	37,2	5,1	56,1	38,7	2,400
94 H E	16,1	58,3	5,4	41,8	52,6	3,605
88 VVDH	21,8	46,7	12,1	54,0	33,7	2,136
781 S D K	24,7	57,3	6,2	49,2	44,5	2,316
79 R M	44,4	37,8	10,3	63,4	26,2	0,851
91 C D P	53,6	19,1	8,6	67,5	23,8	0,543
	30,0	55,3	-	65,9	34,0	1,838
84, M M	11,9	27,1	13,8	55,1	30,9	2,278
81 G B	22,8	38,1	-	67,0	32,9	1,670
110 H A	18,3	55,0	9,5	47,7	42,6	3,000
	55,0	42,6	14,2	48,7	37,0	0,774
90 S N	19,3	24,5	10,9	62,6	26,4	1,322
100 S M	22,5	59,8	5,9	7,43	19,7	2,656
X	23,25	36,92	8,07	53,1	39,75	1,831
SD	± 9,95	±10,5	± 4,04	±13,06	±13,91	±0,788

X Mean value

SD Standard Deviation

TP Total protein

A histogram (Figure 7.9) of the immunoglobulin sub-fraction distribution of the different disease disorders shows the differences that occur.

7.4 DISCUSSION

The isotachophoretic examination of the CSF proteins from a variety of infections and chronic neurological diseases has shown some important differences in the patterns obtained. These differences occur in the total protein profile or in certain regions of the profile - e.g. the immunoglobulin and albumin region. In some cases unidentified components in the profile are greatly elevated.

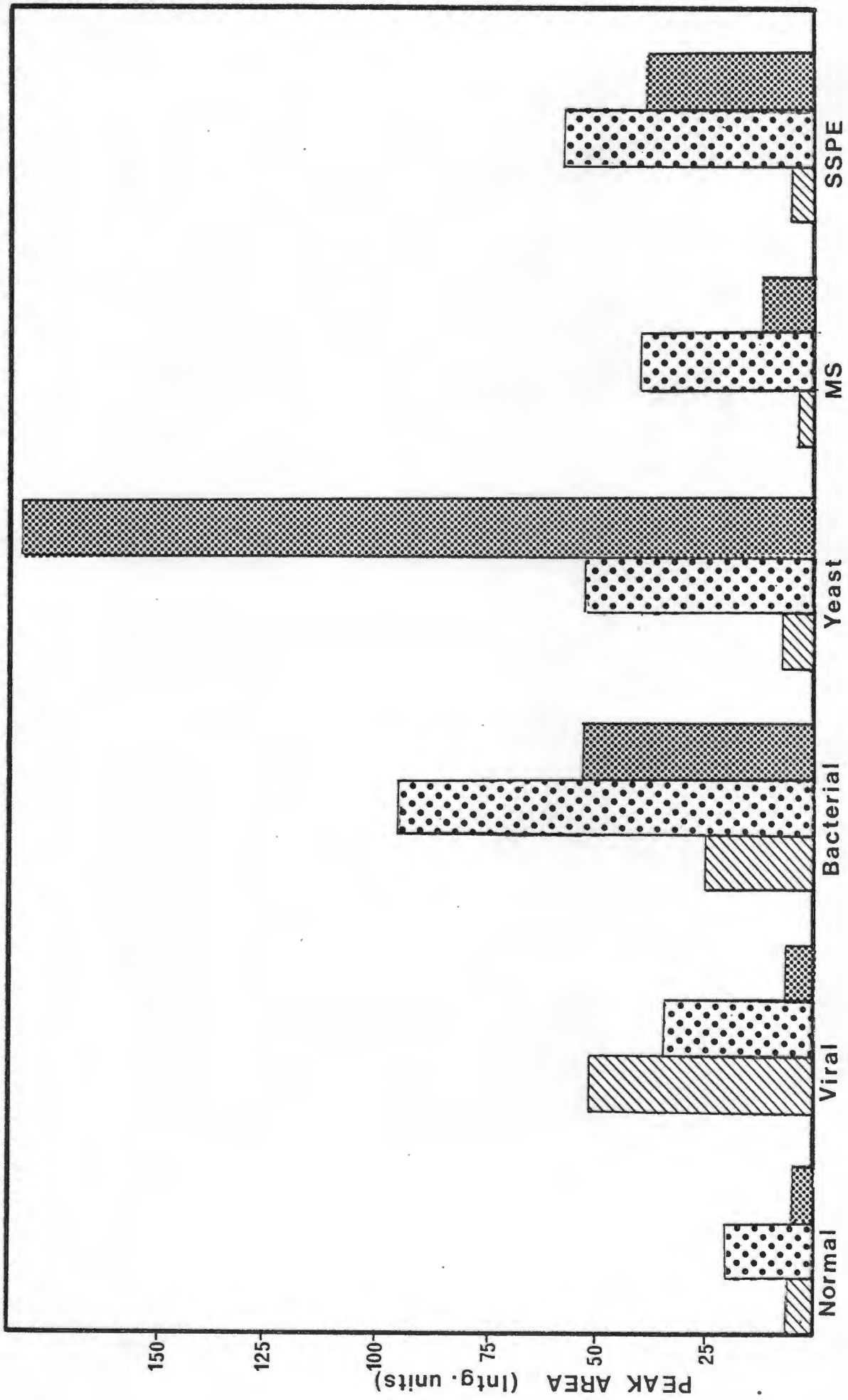
Most workers in this field (Kjellin et al., 1975a, 1975b; Delmotte, 1977; Kjellin and Siden, 1978; Kjellin and Hallander, 1979a, 1979b;) have examined multiple sclerosis CSF samples and have noted differences from the normal pattern. However, in the present study, a variety of other diseases were examined for the first time. These included bacterial and viral meningitis and SSPE CSF samples.

It is difficult to obtain "normal" CSF samples and the source from which these samples were obtained, namely myelography for non-infectious or chronic disorders, was thought to represent the normal CSF pattern profile. This was confirmed by albu-

FIGURE 7.9

Histogram showing the immunoglobulin subfraction distribution in the different diseases.

 subfraction between the amino acid spacers valine and leucine;  subfraction between leucine and β alanine;  subfraction of moility less than β alanine.


PEAK AREA (Intg. units)
150
125
100
75
50
25
Normal
Viral
Bacterial
Yeast
MS
SSPE

min, IgG and total protein analyses, the results of which correspond well to the data of reference values established by Tibbling et al. (1977) and others (See Appendix B). The constant isotachophoretic profile obtained from the 22 samples indicate that no abnormalities are present. This profile may thus be taken as normal. Minor changes in certain peak area distributions were due to individual variations, which one would expect. Two of the FRP, now shown to be folic acid and uric acid, are normal constituents of the CSF.

The IgG/Alb ratio of 0,67 was the upper limit of the ratio obtained by Olsson and Pettersson (1976) at 0,26 - 0,66. All other IgG/Alb ratios determined by isotachophoresis in the diseases examined in this study were above 0,67. Although other proteins - e.g. glycoprotein, prealbumin, orosomucoid and α_1 antitrypsin slot into the albumin peak, using the IgG/Alb ratio the peak was considered to represent albumin only.

In the examination of CSF samples from which virus had been isolated, 60,5% of the samples showed a normal CSF profile. In the 39,5% of cases, an elevated component in the gamma-globulin region, between the amino acid spacers valine and leucine was found.

The time at which the first CSF sample was obtained after onset of symptoms may be an important factor in explaining the presence of this peak. Unfortunately, such information was

not always available. It should be noted, however, that in each of the 104 CSF samples examined, the virus was isolated from the CSF specimen obtained at the initial presentation of the patient. Neither the viral species nor the total cell count could be correlated with the presence of this elevated peak in the protein profile.

In all cases, the overall viral meningitis CSF profile, with or without the abnormal component, was normal. Judging from the size of the albumin peak, the blood-brain barrier was intact. According to Link and Tibbling (1977), the albumin concentration does provide some indication as to the integrity of the blood-brain barrier as synthesis of albumin within the CSF has never been proved to occur. Thus, it must be assumed that the albumin present in the CSF is derived from the serum.

However, the findings of Fryden et al. (1978) suggest that in viral meningitis of mumps origin, the blood-brain barrier is defective. This was not found in the present study. Probably if other methods were employed to examine the integrity of the blood-brain barrier, some slight leakage of serum proteins into the CSF may have been found.

Such methods could include the CSF/serum albumin ratio which Link and Tibbling (1977) feel is more reliable and sensitive. Delmotte (1977) determined blood-CSF barrier permeability by isotachophoresis by recording the albumin and IgG areas of paired serum and CSF samples.

In the present study, there were very few paired CSF and serum samples available to evaluate the above ratios.

The isotachophoretic results of bacterial meningitis CSF samples show a sharply contrasting pattern compared to the patterns obtained from viral meningitis. The number and amount of protein components is greatly increased.

In each case, the causal bacterial agent was isolated from the CSF obtained at the initial presentation of the case. It is clear that even at this early stage of the disease, there is a marked increase in all the proteins. It would thus appear that there is a breakdown in the integrity of the blood-brain barrier with the resultant leakage of serum proteins into the CSF. Bacterial breakdown products are probably also present, but these could not be specifically identified in the complex isotachophoretic profile.

Schuller and Sagar (1981) have characterized this type of change as a transudation with local synthesis of immunoglobulin. The isotachophoretic pattern confirmed that some transudation and, as previously found (Smuts et al., 1982), the increased IgG/Alb ratio of 1,59 indicates that there has been intrathecal synthesis of immunoglobulins.

In all the bacterial cases examined, elevated FRP were noted.

In the cases of meningitis caused by Cryptococcus neoformans an abnormal peak of mobility, less than the slowest gammaglobulin, was found. The possibility of this being a yeast protein cannot be excluded. The presence of cryptococcal antigen in the CSF has been reported and is considered a reliable guide to diagnosis of the encapsulated yeast if not seen by Indian ink stain in the CSF (Fishman, 1980).

In all isotachophoretic studies on multiple sclerosis CSF samples, an increase in the slower immunoglobulin range has been shown (Kjellin et al., 1975a, 1979b; Delmotte, 1977; Kjellin and Siden, 1978; Kjellin and Hallander, 1979b). In the present study an increase was also noted and was confined to the region bounded by the amino acid spacers leucine and β alanine.

The total immunoglobulin content, expressed as a percentage of total protein, was increased from 17,7% in normal to 21,8% in MS. The preferential increase in immunglobulin due to intrathecal production of IgG within the central nervous system was noted as early as 1942 (Kabat et al., 1942) and confirmed in later studies (Lowenthal et al., 1960 and Tourtellotte, 1970).

Oligoclonal bands in the CSF was demonstrated by agar and agarose electrophoresis (Link and Muller, 1971; Thompson et al., 1979; Laurenzi et al., 1980; Chu et al., 1983) in about 90% of the multiple sclerosis cases examined. In isotachopho-

resis, these bands should appear as discrete sharp peaks in the gammaglobulin region, superimposed on a broader peak (Delmotte, 1977). However, such peaks were not observed in the isotachopherograms. Delmotte (1977) suggests that this is due to insufficient amount of these fractions to be detected.

Link (1973b, 1975) investigated the immunoglobulin abnormalities in Guillain-Barré syndrome and noted that the concentrations of IgA and IgM, expressed as a percentage of the total protein, were increased, while this occurred less frequently with IgG. This appeared to be confirmed by the isotachophoretic results obtained in the present study where the IgG concentration was similar to that of the normal values, 16,4% and 17,7% TP respectively. Specific increases in IgA, which separates between the amino acid spacers glycine and valine, were noted in only 3/20 of the cases.

A variation in the GBS CSF protein separation was obtained with both normal and abnormal profiles being recorded. The abnormal profiles usually consisted of an elevated albumin peak and large front running peaks. Fishman (1980) notes that from a variety of studies the variation in the elevation of protein levels is considerable and of importance is the time of lumber puncture. These elevations may be due to blood-brain barrier damage (Link, 1973b) and some of the abnormal isotachophoretic profiles support this observation.

Link et al. (1979) showed specific oligoclonal banding in the CSF in 21% of the GBS cases studied. No distinctive oligoclonal peaks were seen by isotachophoresis.

SSPE is a chronic encephalitis associated with the measles virus and is a fatal disease in childhood and adolescence. The CSF protein levels are usually normal or slightly elevated, but there is a high concentration of gammaglobulins, comprising 20 - 50% of the total protein (Fishman, 1980).

The isotachopherograms of the 37 SSPE examined in this study show a distinctive pattern in all but four cases. The gammaglobulin level expressed as a percentage of the total protein ranges from 20 - 65% and there is a specific elevation in the fraction of mobility less than β alanine. The rest of the protein profile is normal, indicating an intact blood-brain barrier with intrathecal synthesis of immunoglobulins. The IgG/Alb ratio of 1,831 confirms that synthesis must be occurring.

Oligoclonal banding by agarose electrophoresis and isoelectric focusing has been found in all SSPE cases. These bands have been shown to represent antibodies to the measles virus. As previously mentioned, the anodic isotachophoretic system does not appear to separate these specific immunoglobulin clones.

The cationic system which is more suitable for examining the

gammaglobulin region may be a means of detecting these clones isotachophoretically.

The examination of paired SSPE sera (diluted 1:200) have been shown to have a normal serum profile and the increased immunoglobulin peaks are not found (unpublished data).

The isotachophoretic examination of CSF from a variety of infections and chronic neurological diseases, shows differences in the total protein profile and in specific fractions and thus has the potential to be of value in the diagnosis of some of the diseases studied.

CHAPTER VIII

CONCLUSIONS

The isotachophoretic technique has been used in the present study to analyze human cerebrospinal fluid. Fluids from patients with a variety of different neurological diseases were examined, including viral, bacterial and cryptococcal meningitis, probable MS, GBS and SSPE.

The diagnosis of the different diseases were according to accepted clinical and laboratory criteria. The causal agent of meningitis was determined by the growth and isolation of the relevant virus or bacterium from the CSF. The diagnosis of cryptococcal meningitis was by visualization of encapsulated yeasts by Indian ink preparation on direct examination, detection of cryptococcal antigen in the CSF by specific latex agglutimation and the isolation of the yeast on potato dextrose.

Elevated levels of measles antibody in the CSF ($<$ 1:8) is considered to be indicative of SSPE where other antibody levels in the CSF - e.g. polio, are absent or very low. It should be noted that measles encephalitis results in an antibody titre in the CSF of less than 1:4.

It is difficult to obtain normal control CSF samples without subjecting healthy volunteers to lumbar puncture. In the present study, control CSF samples were obtained from patients undergoing myelography for a variety of problems, usually prolapsed intervertebral disc symptoms. To confirm that the CSF samples selected were normal, the concentration of albumin, IgG and total protein were determined. These results were compared with published data.

The total protein concentration determined by the Lowry (Lowry et al., 1951) and Coomassie Brilliant Blue methods (Bradford, 1976), gave similar average results - 33,7 ± 16,3 mg/dL and 34,0 ± 15,8 mg/dL respectively. These results are comparable with the values of Tibbling et al. (1977) of 36,7 ± 6,3 mg/dL, Tourtellotte (1970) of 36,4 mg/dL and Fishman (1980) of 35 mg/dL. Slight discrepancies may be due to the standard used in the present study. Seven parts of a 1% albumin solution and three parts of a 1% IgG mixture were used in the present study, while in most other studies albumin is used as a standard (Tibbling et al., 1977; Eeg-Olofsson et al., 1981).

The albumin and IgG concentration of 19,5 ± 4,6 mg/dL and 1,87 ± 1,13 mg/dL respectively is also within normal published ranges (Ritchie et al., 1973; Ganrot and Laurell, 1974; Olsson and Petterson, 1976; Tibbling et al., 1977; Stevens et al., 1979; Kjellin and Hallander, 1980) (See Appendix B).

The isotachophoretic analysis of the selected normal controls show closely similar profiles with minor individual variations. The samples selected are, therefore, considered to be representative of normal CSF.

Although capillary isotachophoresis lends itself more readily to the analysis of relatively low molecular weight substances (below 3000 daltons) (Hjalmarsson and Baldesten, 1981), larger molecules, like proteins, have been successfully separated (Arlinger, 1974c; Van Kleef et al., 1977; Delmotte, 1977; Gallop and Hambleton, 1979; Hedlund et al., 1979; Kjellin et al., 1975a, 1975b; Del Principe, 1985). This has been due to the addition of long chain polymers to the leading electrolyte to stabilize the highly concentrated protein zones. In complex biological fluids - e.g. serum, CSF and urine, where the proteins have similar mobilities and UV absorbance properties, the addition of spacers - e.g. amino acids and ampholytes, have improved the resolution of the separated zones which are normally in contact with each other.

The concentration of the ampholytes added to the sample is critical if information derived from their specific addition is not counteracted by dilution effects. Due to the large range of mobilities that the ampholytes exhibit within a pH range, a gradient is created with the result that the ampholytes separate with the protein zone, rather than acting as discrete spacers like amino acids. Therefore, the ampholytes dilute the protein zone, resulting in a longer zone with a lower peak height. If too much ampholyte is added, the detection of these diluted zones becomes difficult. In the present study, an ampholyte concentration of 0,02% (v/v) was found to be optimum to separate the CSF proteins without diluting the sample zone with a consequent loss of resolution.

The amino acid spacers not only act as spacers in the protein profile, but also as markers enabling one to readily recognize certain regions demarcated by two amino acids. The addition of leucine to the already accepted amino acid spacers of glycine, valine and β alanine to the CSF (Kjellin et al., 1975a, 1975b; Delmotte, 1977; Zaffaroni et al., 1983; Del Principe, 1985) resulted in the recognition of an elevated component in the immunoglobulin region in 39,5% of the viral meningitis cases examined in the present study.

Quantitative studies on the CSF proteins, albumin and IgG, have confirmed the work of Delmotte (1977) where linear relationships were found between concentration and peak width, peak height and peak area. However, this relationship is not true for all concentrations. Where the zone length is less than the UV slit width, the peak width deviates from linearity. At this concentration, the peak height gives an accurate measurement of concentration. This was first shown by Svoboda and Vacik (1976) for 1,3,5 napthalenetrisulphonic acid.

For the quantitation of protein at low concentrations, the peak area was found in the present study to be a more reliable, accurate and sensitive measurement than peak height or peak width. Svoboda et al. (1983) have also shown that the zone area measurement improves sensitivity and accuracy, especially in complex biological fluids.

One problem found in the present study was that it was not possible to compare the area measurements of the separated CSF proteins with that of a pure substance. For example, the albumin concentration obtained from the CSF profile was greater than that obtained when pure albumin was analyzed. This is, in part, due to other proteins in the CSF separating with the albumin in the MES/Ammediol leading electrolyte system. To overcome this problem it would be necessary to select an electrolyte system with a lower pH in order to separate more efficiently these relatively high mobility proteins. Such a system was not chosen in the present study as all the CSF proteins, especially immunoglobulins, were required to carry a net negative charge in order that they may be detected. Lowering the pH of the electrolyte system would have resulted in the immunoglobulins carrying a net positive charge and, thus, in the anionic system, they would migrate away from the detector.

Quantitation of CSF proteins by isotachophoresis is important if the integrity of the blood-brain (blood-CSF) barrier is to be examined. IgG present in the CSF is either due to transudation from an increase in cerebral capillary permeability, or due to local synthesis of immunoglobulin by lymphocytes which have crossed the capillary walls of the nervous system (Schuller and Sagar, 1981).

Many diseases - e.g. MS, SSPE , progressive rubella encephalitis, cerebrovascular disease and infections of the central nervous system due to syphilis, mumps and measles, are associated with increased levels of gammaglobulins in the CSF.

Normally there is no synthesis of IgG in the CSF and IgG present in CSF is derived from the serum. However, in MS, the rate of IgG synthesis is reported as 32 mg/day (Tourtellotte, 1970). In SSPE the de novo central nervous system IgG measles antibody synthesis rate was between 57 and 145 mg/day (Tourtellotte et al., 1981). This synthesized IgG is characterized by oligoclonal bands that may be detected by electrophoresis, isoelectric focusing and disc electrophoresis (Link, 1973a; Kjellin and Vesterberg, 1974; Takoeka et al., 1976; Johnson, et al., 1977; Link and Tibbling, 1977b; Laurenzi and Link, 1978; Nilsson and Olssen, 1978; Siden and Kjellin, 1978; Laurenzi et al., 1980; Hosein and Johnson, 1981; Iivanainen et al., 1981; Mattson et al., 1981; Kostulas and Link, 1982).

Isotachophoresis has been used to separate IgG in the CSF of MS cases and quantitate the IgG. It has been established that MS patients are slow or cathodic IgG synthesizers - i.e. mobilities less than the amino acid spacer valine (Zaffaroni et al., 1983; Tourtelotte et al., 1982). This correlates with the analysis of CSF from MS patients where abnormal populations of IgG were found in the high-alkaline region by isoelectric focusing (Hosein and Johnson, 1981; Laurenzi and Link, 1979).

Tourtellotte et al. (1982) also established that ACTH and/or steroids had a greater effect in decreasing IgG synthesis when the patients were cathodic synthesizers.

The formula used by Tourtellotte et al. (1982) and Zaffaroni et al. (1983) in calculating the rate of synthesis was based on that of

Tourtellotte et al. (1980) and changed to that recommended by Delmotte.

$$\left[\frac{{}^{>6}\mathrm{IgG}_{\mathrm{CSF}} - \left({}^{<6}\mathrm{IgG}_{\mathrm{CSF}} \,/\, {}^{<6}\mathrm{IgG}_{s} \right)}{{}^{<6}\mathrm{IgG}_{\mathrm{CSF}} + {}^{>6}\mathrm{IgG}_{\mathrm{CSF}}} \right] \mathrm{IgG}_{\mathrm{CSF}} \times 5$$

Where ${}^{<6}\mathrm{IgG}$ are the areas under the first 6 IgG peaks in the isotachopherogram (fast IgG) and ${}^{>6}\mathrm{IgG}$ are the areas under the remaining slow IgG peaks.

In order to determine the synthesis rates, it is necessary to have paired CSF and serum samples. Such samples were not available in the present study and thus this ratio could not be used.

Although IgG synthesis rates could not be determined, the integrity of the blood brain barrier could be analyzed. Where the blood-brain barrier is normal, as indicated by the albumin level, an increase in IgG would suggest synthesis within the central nervous system.

The degree of transudation or integrity of the blood-brain barrier is estimated from the increase in CSF albumin concentration as albumin has a high rate of diffusion due to its relatively low molecular weight; it forms a large proportion of the total protein in the CSF; fluctuations in concentration are non-specific as albumin has no known pathological role; and it may be precisely measur-

ed (Schuller and Sagar, 1981). These authors have also established that where the CSF albumin concentration is normal, there is no correlation between increased serum IgG and CSF IgG. Thus, the increase in CSF IgG in this situation is due to local synthesis only.

The CSF IgG/protein ratio or the CSF IgG/albumin ratio may be used to demonstrate a selective increase in IgG due to synthesis within the central nervous system (Link and Tibbling, 1977b). However, in some cases this synthesis may be missed if the serum albumin and IgG levels are not considered (Link and Tibbling, 1977a). The IgG index (Link and Tibbling, 1976) takes into account both serum IgG and the occurrence of blood-brain barrier damage and is expressed as :

$$\text{IgG index} = \frac{\frac{\text{IgG}}{\text{CSF}} \times \frac{\text{Albumin}}{\text{serum}}}{\frac{\text{IgG}}{\text{serum}} \times \frac{\text{Albumin}}{\text{CSF}}}.$$

The normal IgG index ratio as determined by Link and Tibbling (1976) and Olsson and Pettersen (1976) are 0,34-0,58 and 0,66 respectively.

In the present study, the CSF IgG/albumin ratio for control CSF cases as determined by isotachophoresis was 0,674. In all the

chronic and/or infectious neurological diseases examined, this ratio was increased to 0,802 and 1,077 in viral meningitis; 1,045 in MS; 0,723 in GBS and 1,831 in SSPE.

These values indicate that there is IgG synthesis within the central nervous system.

Qualitative analysis of the CSF isotachophoretic protein profile of bacterial and cryptococcal meningitis has shown that there is severe damage to the blood-brain barrier with transudation of serum proteins into the CSF. In these cases there may be local IgG synthesis as well. This has been termed a "meningitic" profile (Schuller et al., 1978).

Delmotte (1977), used the isotachophoretic formula to indicate blood-brain barrier permeability:

$$\frac{A_{n(CSF)}}{10f\ (CSF)} \times \frac{1}{A_n\ (serum)} \times 1000$$

Where A_n is the area under the peak of fraction n and f the concentration factor. Delmotte (1977) was able to distinguish normal blood-brain barrier permeability with local synthesis from abnormal blood-brain barrier permeability and definite intracerebral synthesis.

Viral meningitis also produces a meningitic profile with an increase in total protein due to a damaged blood-brain barrier (Ursing, 1965; Fryden et al., 1978; Fishman 1980; Schuller and Sagar 1981). However, by isotachophoresis, the CSF protein profile could not be considered abnormal - i.e. presence of transudation as shown by an elevated albumin peak. This may, in part, be due to the CSF samples obtained in the present study being taken at an early stage of the disease. Paired serum and CSF samples would have been useful for Delmotte's isotachophoretic method of determining blood-brain barrier permeability to show some transudation.

The qualitative examination of the CSF protein profiles of several other diseases differed from the controls. These differences were usually confined to the immunoglobulin region - e.g. MS, SSPE. GBS produced a wide variation of separation profiles so that no single pattern could be ascribed to this disease. The FRP region also varied with increases noted in many of the diseases.

With viral meningitis due to a variety of different viruses - e.g. $ECHO_4$, Coxsackie B_5 and mumps, two different patterns emerged.

In about 60% of the 104 cases examined, the protein profile was normal. However, in the other 40%, a component between valine and leucine was increased. Attempts to identify this component have shown that it is not IgM. As it is absorbed to Protein A, it is of the IgG class. Protein A specifically binds the human IgG subclasses 1, 2 and 4 and not IgG_3 (Kronvall and Williams, (1969).

Beck (1981) has suggested that viral activity is associated with the IgG_3 subclass, but this could not be proved in the present study.

Associated with infection and cellular damage is the acute phase response which results in the increase of acute phase proteins, including C reactive protein (CRP) (Kushner, 1982). This protein has been found to be considerably enhanced in bacterial meningitis and some viral meningitis cases (Sindic et al., 1984). The physiochemical properties pI 7,9 and molecular weight of 110 000-144 000 daltons (Baltz et al., 1982; Oliveria et al., 1977) suggest that it could separate in the immunoglobulin region.

Studies with Limulus CRP proved negative as the addition of this protein did not specifically increase the peak between valine and leucine (unpublished observations).

It was subsequently found that this CRP has a pI of between 4 and 5 using the titration method of Righetti et al., (1978) (unpublished observations). Thus, although Limulus CRP does not specifically increase the peak between valine and leucine, human CRP should be added to prove or disprove this idea.

One of the reasons for the elevation of this component in only 40% of the cases examined may be due to the time at which the CSF is taken after onset of symptoms. Such information was rarely availa-

ble in the present study, so this parameter could not be fully investigated.

However, in one documented case, a CSF sample taken 48 hours after onset of symptoms suggestive of meningitis - i.e. headache, nausea and neck stiffness (Echo 4 subsequently isolated from the CSF), a greatly elevated component was found in the position between valine and leucine.

Ursing (1965) examined the CSF of 131 patients in the acute phase of viral meningoencephalitis, by immunoelectrophoresis for five proteins - three plasma proteins not normally occurring in the CSF namely α_2 macroglobulin, fibrinogen and β_1 lipoprotein. γ_1 macroglobulin and gammaglobulin which occur in the CSF in low concentrations were also examined. He found that at the early stage of the disease, the two immunoglobulins, γ_1 macroglobulin and gammaglobulin, occur simultaneously in the CSF with one or more of the transproteins, probably due to a disturbance in the blood-brain barrier. Two to three weeks after onset, the β_1 lipoprotein and fibrinogen had disappeared to a greater extent than α_2 macroglobulin and γ_1 macroglobulin, indicating that the blood-brain barrier had recovered.

The possibility that one of these proteins may be responsible for the elevated peak seen by isotachophoresis must still be investigated, although α_2 macroglobulin has been shown to separate near the albumin.

This component is unlikely to be a viral protein, as the elevation is not restricted to a single viral group.

Bacterial meningitis showed a markedly different protein profile from that of viral meningitis. The initial stage of the disease is characterized by a large protein content including albumin which indicates that there is severe damage to the blood-brain barrier.

Cryptococcal meningitis showed an interesting separation pattern where a distinct highly UV-absorbing component of mobility less than the slowest immunoglobulin was found. Identification of this component was not performed, but the possibility exists that it may be a cryptococcal antigen. The titres of the cryptococcal antigen in the CSF of all three cases were greater than 1:8. The total protein concentration was also increased.

Examination of the CSF from probable MS patients showed an increase in the immunoglobulin of mobility intermediate to leucine and alanine. This confirms previous reports (Kjellin et al., 1975a, 1975b; Delmotte, 1977; Tourtellotte et al., 1982; Zaffaroni et al., 1983). The total protein content is either normal or slightly elevated and may be ascribed to increased endothelial cell permeability associated with demyelinative lesions (Fishman, 1980).

The FRP are also elevated in a number of the MS cases and in many other neurological diseases of unknown origin (unpublished observa-

tions). One of the FRP has been identified as uric acid, a normal constituent of the CSF (concentration 0, 25 mg/dL (Fishman, 1980)). Elevated levels of CSF uric acid are due to increased neuronal degeneration associated with increased nucleic acid catabolism. Such elevations have been found in uremia, meningitis (Merritt and Fremont-Smith, 1938), increasing age (Praetorius, 1957), cerebral atrophy (Young and Crampton, 1974) and alcohol withdrawal states (Carlsson and Dencker, 1973).

A CSF sample from a patient with suspected meningitis, with a CSF uric acid concentration of 4 mg/dL and an elevated component in the FRP3 region, was incubated with uricase. On re-examination of the CSF by isotachophoresis, the profile showed a reduction of the uric acid peak and the presence of the breakdown product, allantoin.

Also identified as a FRP was folic acid. This occurs in the reduced form in the CSF as methyltetrahydrofolate (Chanarin, 1980; Spector, 1977; Fishman, 1980) at a concentration 2,5-5 times higher than in the serum. There is a specific folate saturable active transport system located in the choroid plexus which maintains these levels even if there is a low concentration of folate in the plasma (Spector and Lorenzi, 1975; Spector, 1977). However, in diseases - e.g. meningitis, the normal CSF/plasma vitamin gradient is diminished or abolished, partly due to interference with the

transport system. Local folate deficiencies in the brain may be the cause of unexplained signs and symptoms of meningitis (Spector, 1977).

The isotachophoretic analysis of the large number of CSF samples examined, has shown that the folic acid level is constantly maintained. However, bacterial meningitis appears to be an apparent exception.

Decrease in serum folate levels have been found in MS and other diseases - e.g. peripheral neuropathy, myelopathy, geriatric patients and other organic brain diseases (Isager, 1970). However, such deficiencies would be unlikely to affect the CSF levels.

Serum folate deficiency has also been associated with depression and neuropsychiatric signs. This is due to the presence of low levels of the brain biogenic amines - e.g. 5-hydroxyindoleacetic acid (Botez et al., 1982).

It should be noted that methotrexate, a folic acid antagonist and a drug used in the treatment of acute lymphocytic leukaemia (ALL), also separates in the FRP region.

Del Principe et al. (1985) in their examination of the CSF from children with ALL, treated with a variety of drugs, including methotrexate, noted the presence of an unidentified rapidly moving component. The possibility exists that this component may be methotrexate.

A distinctive CSF protein separation profile was found in almost all (33/37) of the SSPE cases examined. Although the albumin peak was normal, indicating an undisturbed blood-brain barrier, the slow immunoglobulin region was greatly elevated - i.e. immunoglobulins of mobility less than leucine. The IgG/albumin ratio of 1,802 indicates that there is intrathecal IgG synthesis. It has been shown that from 10-90% of the total IgG in the CSF has anti-measles virus activity (Mehta et al., 1977; Mehta et al., 1982; Vandvik et al., 1976).

Attempts to determine the specificity of the elevated immunoglobulin peaks are under way at present. These include absorption studies to remove measles antibody in the CSF by incubating the sample, either with a measles antigen coated polystyrene bead or linking the measles antigen to cyanogen bromide Sepharose matrix and performing chromatography.

It has been established that the isotachophoretic separation of CSF results in two distinct UV-absorbing regions - the FRP region and the main protein region. The FRP region which consists of highly mobile substances (mobility greater than the MES ion but less than the chloride ion) with molecular weights less than 1000 daltons were not detected when the CSF was dialyzed against distilled water (Exclusion chromatography also confirmed this finding (unpublished observations)). These procedures also removed sodium chloride which, in turn, resulted in the FRP migrating zone electrophoreti-

cally in an "out of stack" configuration (Mikkers and Everaerts, 1981) in the MES/Ammediol leading electrolyte.

The analysis of the FRP in the MES system with sodium chloride from the sample showed that these peaks were equivalent to zones in the capillary of approximately 1 mm in length. 10 µL CSF in a 0,5 mm diameter capillary occupies a length equivalent to 50 mm. If these FRP components were migrating zone electrophoretically, they would also occupy an equivalent or even longer length due to diffusion. This confirms that the FRP are being concentrated.

The chloride ion present in the CSF as sodium chloride was found to move through the MES/Ammediol leading electrolyte and take over as the leading ion with the fraction of the overtaken MES ion now acting as a spacer. The MES slots into a position between the FRP and the main protein region of the CSF.

Chloride ion is present in the CSF at a concentration of 125 mM. This concentration is considerably higher than the leading electrolyte concentration of 5 mM. However, isotachophoresis does take place although separation conditions are not ideal.

Thus, the presence of salts in biological material greatly influences the separation and resolution of these complex samples and their role must be taken into consideration when designing electro-

lyte systems. Although isotachophoresis under such conditions may not be ideal, these highly mobile ions - e.g. chloride and phosphate (see Figure 6.11) are useful in the simultaneous examination of high and low mobility compounds in single sample.

APPENDIX A

DERIVATION OF THE KOHLRAUSCH REGULATING FUNCTION

The derivation of the Kohlrausch regulating function is given below:

To calculate the concentration and the relationship between the leading and terminating electrolytes one must assume the following conditions (Everaerts et al., 1973).

(a) balance of current - current is constant throughout the system.

(b) balance of mass - the concentration of the counterion in one zone is equal to that in another zone.

(c) principle of electroneutrality - the number of positive and negative charges in one zone is equal.

(d) equilibrium equations - acid and base equilibria determine the partial ion concentration.

(e) negligible diffusion effects.

(f) no activity coefficients need to be included as solutions are dilute.

(g) area through which the current passes is constant.

(h) negligible electroendosmosis effects.

(i) no hydrodynamic flow exists.

The net effective mobility (meff) of an ion (i) is defined as the product of the ionic mobility (m) and the degree of dissociation (x).

$$m_{eff} = \sum m_i x_i$$

But under identical experimental conditions, the ionic mobility remains constant. The effective mobility can, therefore, be described as

$$m_{eff} = \text{constant } x_i$$

The electrical conductivity (λ) of a solution of ions is a function of the concentration of the ions (ci), the mobility (mi) and the elementary charge (z_i). E is the charge of an electron.

$$\lambda = E \sum c_i m_i z_i$$

The average velocity of migration Si in a voltage gradient V is:

$$Si = V.m_i.x_i$$

In the isotachophoretic system the current (I) is constant and velocities (S) of the leading ion (α) and terminating ion (γ) are the same.

Thus

$$\frac{m\alpha \,.\, x\alpha}{\sum c\alpha \; m\alpha \; z\alpha} = \frac{m\gamma \,.\, x\gamma}{\sum c\gamma \; m\gamma \; z\gamma}$$

A common buffering counterion (β) is also found in the system. Therefore

$$\frac{m\alpha \cdot x\alpha}{(c\alpha \cdot m\alpha \cdot z\alpha)+(c\beta \cdot m\beta \cdot z\beta)} = \frac{m\gamma \cdot x\gamma}{(c\gamma \cdot m\gamma \cdot z\gamma)+(c\beta \cdot m\beta \cdot z\beta)}$$

But satisfying the condition of electroneutrality, the equation can be written in the following manner:

$$\frac{c\alpha \cdot x\gamma}{c\gamma \cdot x\alpha} = \frac{z\gamma \cdot m\alpha \ (m\gamma - m\beta)}{z\alpha \cdot m\gamma \ (m\alpha - m\beta)}$$

This is the Kohlrausch regulating function.

APPENDIX B

PROTEIN DETERMINATIONS OF "NORMAL" CSF SAMPLES

B.1 INTRODUCTION

There is difficulty in obtaining normal control CSF samples without subjecting healthy individuals to lumbar puncture procedures. The source of normal control CSF samples in this study was from patients undergoing myelography for leg and neck pain, probably associated with disc problems.

To confirm that these CSF samples had normal protein parameters, the total protein concentration was determined using the Lowry method (Lowry et al., 1951) and the Coomassie Brilliant Blue method (Bradford, 1976). The albumin and IgG concentrations were also determined by radial immunodiffusion (RID)

The total protein concentration of the normal controls selected in this study was 34 mg/dL and 33,7 mg/dL using the Coomassie Brilliant Blue and Lowry methods respectively (See Chapter VII - Table 7.1).

The albumin and IgG concentrations as determined by radial immunodiffusion were 19,5 mg/dL and 1,87 mg/dL respectively (Chapter VII - Table 7.1).

These results compared favourably with published data (Table B.1, Table B.2 and Table B.3), although the IgG concentration in this study is generally lower than the values obtained in other studies.

The description of the Lowry and Coomassie Brilliant Blue methods for the determination of total protein in the CSF is given below. A comparison is made between different protein standards - i.e. IgG, albumin and a 7+3 mixture of albumin and IgG. The RID method is also described.

TABLE B.1 - NORMAL CSF TOTAL PROTEIN CONCENTRATIONS

Reference	Subjects	Number	Mean	Range
Ursing (1965)	Patients	21	41,9	29,9-53,9
Hartley et al. (1966)	Patients	14	34,0	16,0-48,0
Gilland (1967)	Volunteers	11	36,0	24 -49
Laffin (1970)	Patients	100	32,0	17,1-46,9
Nerenberg and Prasad (1975)	Patients	35	28,2	-
Tibbling et al. (1977)	Patients	93	43,3	36,7-52,6
Nerenberg et al. (1978)	Patients	-	30,0	15,0-45,0
Stibler (1978)	Volunteers	32	44,0	23,0-68,0
Eeg-Olloffson et al. (1981)	Patients	105	36,4	29,5-41,2

TABLE B.2 - NORMAL CSF ALBUMIN CONCENTRATIONS

Reference	Subjects	Number	Mean	Range
Ritchie et al. (1973)	Patients	100	18,6	13,4-23,7
Ganrot & Laurell (1974)	Patients	54	30,4	10,0-60,0
Olsson & Petterson (1976)	-	-	20,5	-
Tibbling et al. (1977)	Patients	93	20,6	17,0-24,2
Stevens et al. (1979)	Patients	50	19,46	10,6-31,3
Kjellin & Hallander (1980)	Patients	15	17,81	-

TABLE B.3 - NORMAL CSF IgG CONCENTRATIONS

Reference	Subjects	Number	Mean	Range
Hartley et al. (1966)	Patients	14	2,3	1,0- 3,11
Laffin (1970)	Patients	100	3,2	1,4- 5,6
Ritchie et al. (1973)	Patients	100	3,3	-
Ganrot & Laurell (1974)	Patients	54	2,06	0,8- 6,4
Nerenberg & Prasad (1975)	Patients	35	4,54	-
Olsson & Petterson (1976)	-	-	1,92	-
Tibbling et al. (1977)	Patients	93	2,3	1,7- 3,7
Nerenberg et al. (1978)	Patients	35	4,6	0,8- 8,4
Mingioli et al. (1978)	Volunteers	31	1,88	0,6- 5,85
Stevens et al. (1979)	Patients	50	2,33	1,14-3,90
Kjellin & Hallander (1980)	Patients	15	1,54	-

B.2 LOWRY PROTEIN DETERMINATION

This method is often used for protein determinations. It is a sensitive method detecting as little as 4 ng/µL protein (Lowry et al., 1951).

B.2.1 Solutions

A working potassium/sodium tartrate copper solution was made by mixing equal volumes of 1% (m/v) copper sulphate ($CuSO_4.5\ H_2O$) (BDH Chemicals Ltd., England) and 2% (m/v) potassium/sodium tartrate (COOK. CH (OH). CH(OH). COO.Na. $4H_2O$) (BDH Chemicals Ltd., England) made up in distilled water.

A 2% (m/v) sodium carbonate ($Na_2\ CO_3$) (Sigma Chemical Co., U.S.A.) solution was prepared in 0,1N sodium hydroxide (NaOH) (Sigma Chemical Co., U.S.A.). A fresh solution was prepared whenever required.

The alkaline copper solution required in the protein determination was prepared by mixing 50 mL $Na_2\ CO_3$ solution with 1 mL $CuSO_4$ K/Na tartrate solution.

The Folin and Ciocalteu's phenol reagent (BDH Chemicals Ltd., England) was diluted 1:2 with distilled water before use.

B.2.2 Method

0,1 mL CSF was mixed with 0,5 mL alkaline copper solution. This mixture was allowed to stand for 10 minutes at room temperature (20°C). 50 µL of the diluted Folin and Ciocalteu solution was added rapidly and mixed for 10 seconds in a vortex mixer. After 30 minutes at room temperature the absorption of the solution was measured in a spectrophotometer (Perkin Elmer Double Beam spectrophotometer) at wavelength 500 nm. The cell path length was 10 mm.

Standard curves of dilutions of 1% (m/v) bovine serum albumin (BSA) (Sigma Chemical Co., U.S.A.), 1% (m/v) human immunoglobulin G (IgG) (Sigma Chemical Co., U.S.A.), and a mixture of 1% (m/v) albumin and 1% (m/v) IgG in a ratio 7+3 were used to determine the standard curve most appropriate for the examination of CSF.

Johnson and Lott (1978), using the Coomassie Blue method (described below) suggested that inaccurate results would be obtained if only albumin or IgG were used to prepare a standard curve. A solution more closely resembling CSF would minimize this source of error and give more accurate results. Normal CSF consists of approximately 70% albumin and the rest immunoglobulins.

B.2.3 Results

The absorbance readings of two experiments, (A and B), at 500 nm of dilutions of 1% BSA, 1% human IgG and a mixture of BSA and IgG in the ratio 7+3 are given in Table B.4.

Table B.5 is the computed "best fit" results (least squares method) for BSA, IgG and BSA+IgG in the two different experiments, A and B.

Standard curves of the three protein solutions are given in Figure B.1.

TABLE B.4 - LOWRY PROTEIN DETERMINATION. ABSORBANCE READINGS OF BSA, IgG AND 7+3 RATIO OF BSA AND IgG. TWO EXPERIMENTS A AND B.

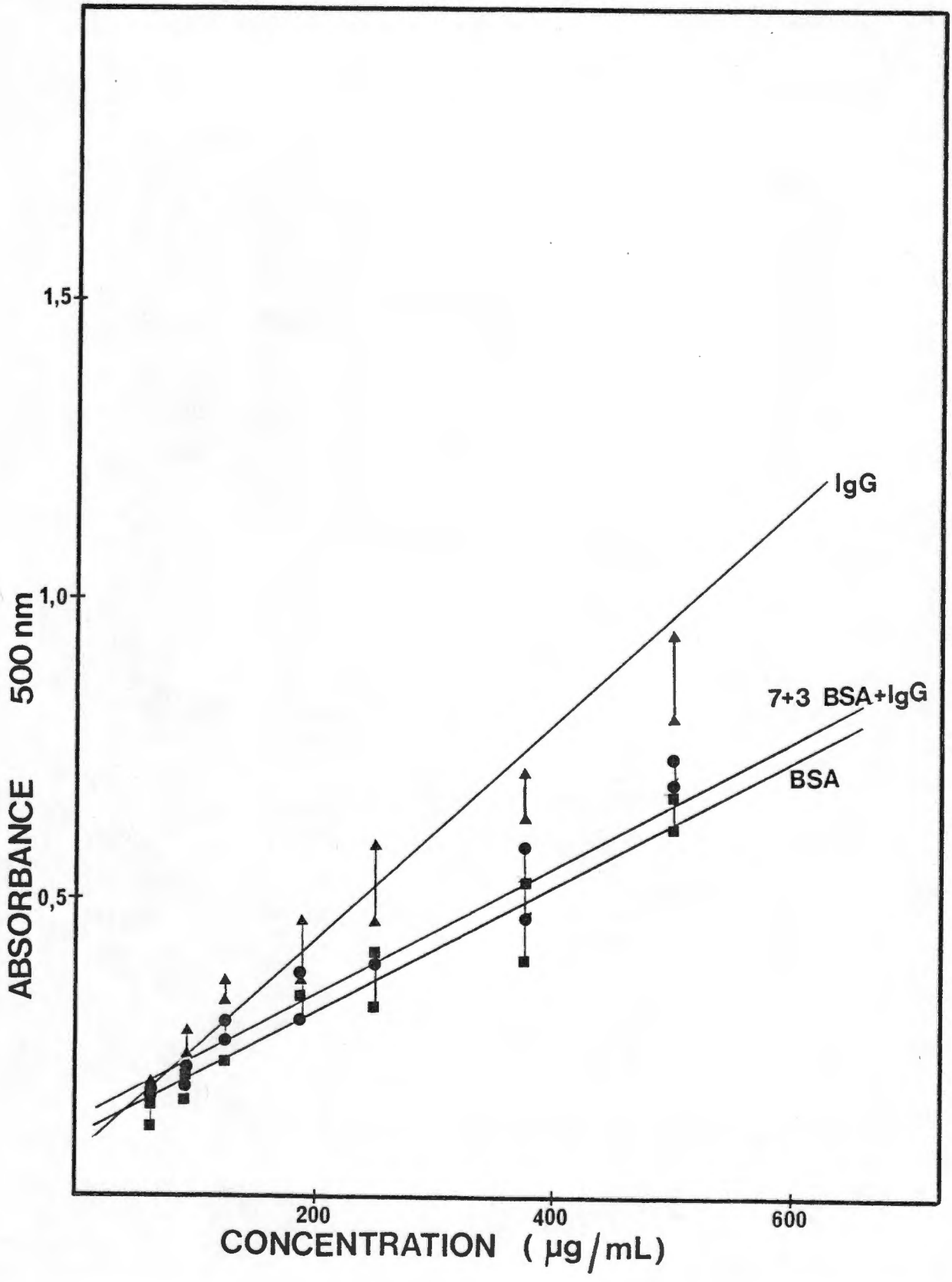
PROTEIN CONC (µg / mL)	ABSORBANCE AT 500 nm					
	BSA		IgG		7+3 BSA+IgG	
	A	B	A	B	A	B
62,5	0,128	0,160	0,194	0,185	0,158	0,180
93,75	0,170	0,201	0,232	0,280	0,179	0,220
125,0	0,273	0,238	0,360	0,328	0,298	0,261
187,5	0,295	0,341	0,365	0,463	0,294	0,376
250,0	0,323	0,405	0,460	0,587	0,395	0,392
375,0	0,403	0,528	0,634	0,708	0,468	0,589
500	0,675	0,625	0,800	0,940	0,729	0,685
750	0,845	0,895	1,800	1,23	0,858	0,905
Correlation Coefficient	0,9823	0,9966	0,9584	0,9932	0,9813	0,9927
Y-Intercept	0,0868	0,1169	-0,0144	0,1523	0,1153	0,1388
Slope	0,0010	0,0010	0,0021	0,0014	0,0010	0,0010

TABLE B.5 - THE AVERAGE "COMPUTED BEST FIT" ABSORBANCE READINGS FOR BSA, IgG AND 7+3 BSA AND IgG AS DETERMINED BY THE LOWRY METHOD

PROTEIN CONC. (μg/mL)	ABSORBANCE AT 500 nm		
	BSA	IgG	7+3 BSA+IgG
50	0,1538	0,1591	0,1799
100	0,2058	0,2495	0,2327
250	0,3618	0,5204	0,3912
500	0,6218	0,9718	0,6554

FIGURE B.1

Standard curves of various concentrations of human IgG (▲—▲), BSA (■—■) and a 7+3 mixture of BSA and IgG (●—●) as determined by the Lowry protein method.


1,5
1,0
0,5
ABSORBANCE 500 nm
IgG
7+3 BSA+IgG
BSA
200
400
600
CONCENTRATION (µg/mL)

If one used only pure IgG as a standard, one would underestimate the protein concentration by approximately 25% as compared to a 7+3 BSA and IgG mixture. There is an overestimation of 11% if only albumin were used as a standard.

It should be noted that the colour intensity of the Lowry method is determined by both proteins and amino acids in the solution.

B.3 COOMASSIE BRILLIANT BLUE G250 PROTEIN DETERMINATION

This is a rapid, sensitive method which is easily performed. It makes use of a colour change of the dye Coomassie Brilliant Blue G250, which is brown in the leuko form at low pH and turns blue in the presence of protein due to protein-dye binding. According to Bradford (1976), the method is four times more sensitive than the Lowry method. It cannot measure peptides of less than 3000 daltons (Sedmark and Grossberg, 1977). Thus, amino acids and short polypeptides are not included in the total protein estimation. An added advantage is the simplicity of the test as only one reagent is required and it is not necessary to incubate the mixture. A disadvantage is that there are a number of detergents -e.g. Triton X100; denaturing agents - e.g. SDS, 2 mercaptoethanol; strong alkaline solutions; Tris; acetic acid; sucrose; glycerol and hemosol,

which interfere with the colour change. Ampholytes may also affect the dye reagent (Bradford, 1976). However, the effect may be nullified by adding the substance to the blank at the correct concentration.

B.3.1 Stock Dye Reagent

10 mg Coomassie Brilliant Blue G250 (also known as Page Blue G90) (BDH Chemicals Ltd., England), was dissolved in a solution composed of 5 mL absolute ethanol and 10 mL 88% orthophosphoric acid (H_3PO_4) (Hopkin and Williams Ltd., England). This solution was then diluted to 100 mL with distilled water.

B.3.2 Method

To 100 µL protein solution (in this case, dilutions of 1% BSA, 1% IgG and mixture of 1% BSA and 1% IgG in ratio 7+3), was added 3 mL of the stock dye reagent. These solutions were mixed for 10 seconds on a vortex mixer and may be measured immediately in a spectrophotometer at 595 nm. It was unnecessary to read at 465 nm as well because the double beam spectrophotometer automatically subtracts the blank control.

B.3.3 Results

Table B.6 shows the absorbance readings at 595 nm of the dilutions of 1% BSA, 1% IgG and mixture 7+3 BSA+IgG. The

results of two experiments, A and B, are given.

The average computed "best fit" absorbance readings for the three different proteins in the two experiments are given in Table B.7. The least squares method was used.

Figure 3.2 illustrates the standard curves obtained for the three proteins examined by this method.

TABLE B.6 - COOMASSIE BRILLIANT BLUE G250 PROTEIN DETERMINATIONS OF BSA, IgG AND 7+3 MIXTURE OF BSA and IgG FOR STANDARD CURVES. A AND B REPRESENT TWO DIFFERENT EXPERIMENTS

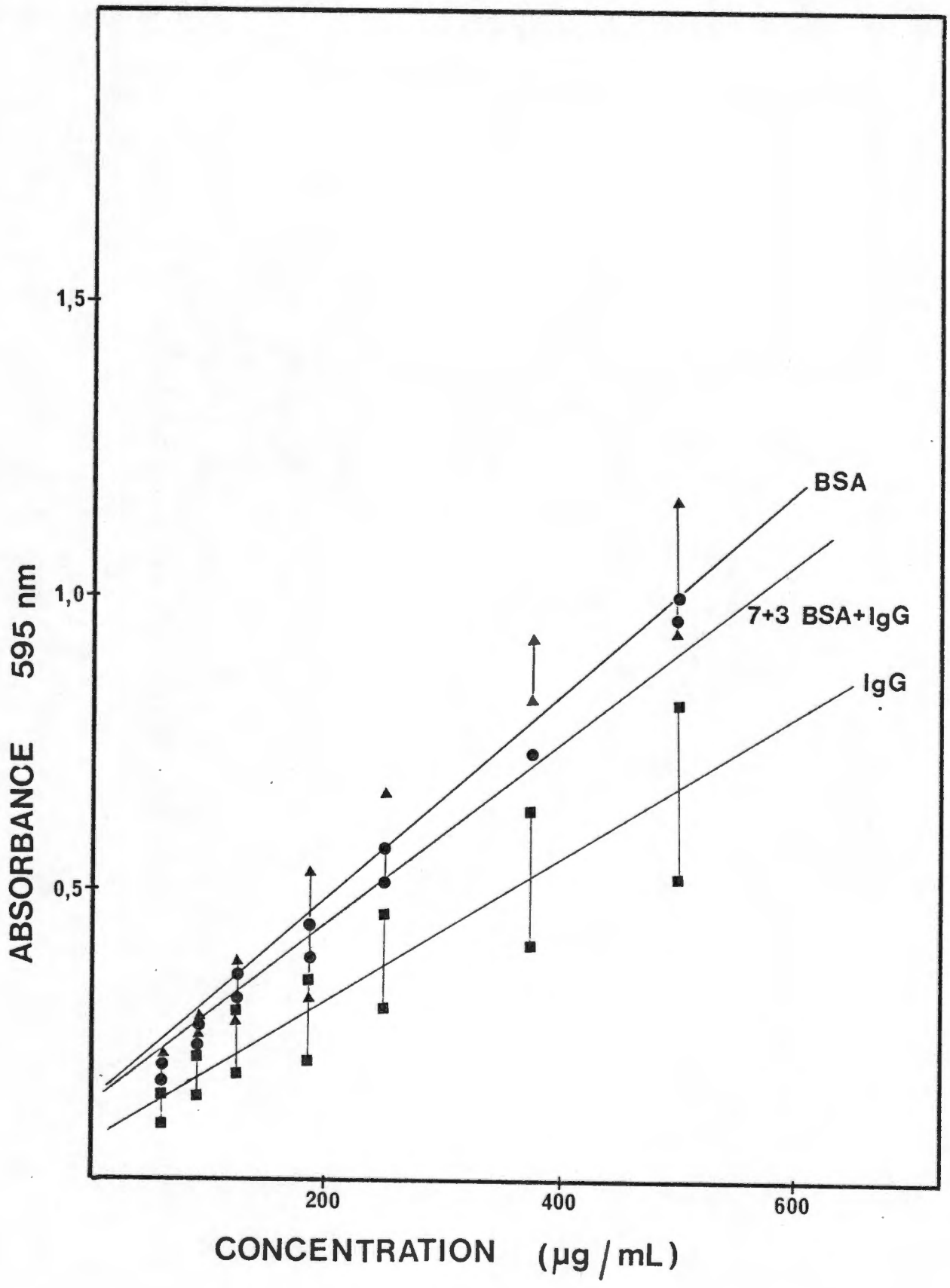
PROTEIN CONC. (µg/mL)	ABSORBANCE AT 595 nm					
	BSA		IgG		7+3 BSA and IgG	
	A	B	A	B	A	B
62,5	0,215	0,151	0,151	0,095	0,200	0,161
93,75	0,280	0,258	0,210	0,150	0,271	0,230
125,0	0,372	0,280	0,288	0,182	0,358	0,315
187,5	0,532	0,310	0,340	0,208	0,435	0,387
250,0	0,663	0,572	0,458	0,294	0,570	0,519
375,0	0,928	0,825	0,630	0,403	0,730	0,732
500	1,160	0,938	0,815	0,518	1,02	0,960
750	1,48	1,250	1,10	1,855	1,25	0,190
Correlation Coefficient	0,9926	0,9837	0,9972	0,9949	0,9912	0,9907
Y-Intercept	0,1847	0,0933	0,0094	0,0289	0,1475	0,1110
Slope	0,0018	0,0016	0,0014	0,0010	0,0015	0,0015

TABLE B.7 - THE AVERAGE "COMPUTED BEST FIT" ABSORBANCE READINGS FOR BSA, IgG AND 7+3 BSA AND IgG AS DETERMINED BY THE COOMASSIE BRILLIANT BLUE METHOD

PROTEIN CONC. (µg/mL)	ABSORBANCE AT 595 nm		
	BSA	IgG	7+3 BSA+IgG
50	0,225	0,220	0,206
100	0,311	0,183	0,283
250	0,570	0,365	0,516
500	1,00	0,670	0,903

FIGURE B.2

Standard curves of various concentrations of human IgG (■—■), BSA (▲—▲) and a 7+3 mixture of BSA and IgG (●—●) as determined by the Coomassie Brilliant Blue method.


1,5
1,0
0,5
ABSORBANCE 595 nm
BSA
7+3 BSA+IgG
IgG
200
400
600
CONCENTRATION (µg / mL)

With pure IgG used as a standard, one overestimates the protein concentration by approximately 31%. This compares with Johnson and Lott (1978) who find an overestimation of between 32-43%. With pure albumin as standard, the error is an underestimation of protein by 8,7-11%. This, again, compares favourably with the results of Johnson and Lott (1978) of an underestimation of 8-15% when compared with the mixture of BSA and IgG in ratio 7+3.

It is of interest to note that the results obtained for the standard curves of BSA and IgG, as determined by the Coomassie Blue method, are opposite to that obtained with the Lowry method where one overestimates the protein concentration when albumin is used and underestimates the result if pure IgG is used. The reason for this is unknown.

In both the Folin reaction (Lowry et al., 1951) and the Coomassie Blue Method (Bradford, 1976), the amount of colour produced varies with different proteins. Pierce and Suelter (1977), however, suggest that there is no colour variability in the Lowry method due to different proteins. In their study they analysed seven different proteins: BSA, cytochrome C, trypsin, chymotrypsin, pep-

synogin lysozyme and chymotrypsinogen. In the present study, the standard curves of albumin, IgG and the mixture, obtained by the Lowry method, do differ and this does not support the work of Pierce and Suelter. This variability is also demonstrated using the Coomassie Blue G250 protein determination method. According to Pierce and Suelter (1977), the variability may be due to the amount of dye bound.

B.4 RADIAL IMMUNODIFFUSION FOR ALBUMIN AND IgG DETERMINATIONS

The single immunodiffusion technique of Mancini et al. (1965) was used. This is a simple and specific method for the identification and quantitation of serum and CSF proteins. The principle of radial immunodiffusion (RID) is based on the precipitin reaction in which the specific antibody is incorporated into a buffered agarose gel. Five µL of the antigen sample (CSF), containing the IgG and albumin to be measured, is placed in a well punched into the gel. The plate is incubated for 18-24 hours at room temperature for the antigen to diffuse outwards into the gel. At a certain distance from the well, the concentration of the antigen reaches equivalence with the antibody in the gel, and a precipitin ring is formed round the well. The diameter of the circle is proportional to the amount of antigen originally present. Thus, by comparing

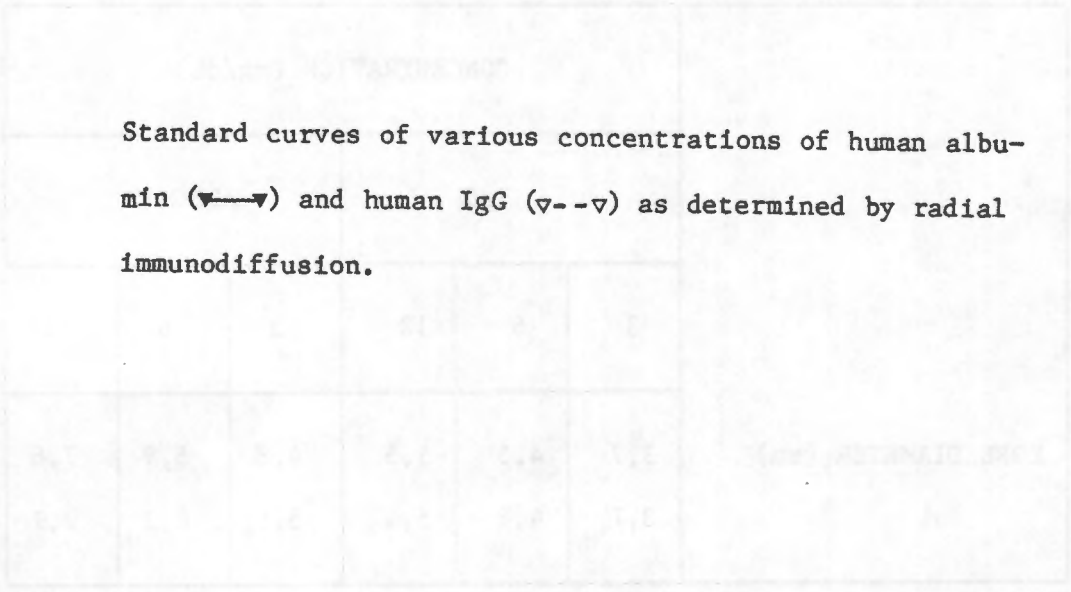
the diameter of zones of known concentrations of antigen with the diameter of an unknown, the concentration of the unknown can be determined.

In all determinations, the RID system for human IgG and albumin of ICL Scientific (Fountain Valley, California, U.S.A.) was used. This is designed to quantitate low concentrations of albumin and IgG - e.g. in CSF, as low reference IgG and albumin sera concentrations are used. An example of the results and standard curve for albumin and IgG are given in Table B.8 and Figure B.3. However, it is essential to establish a reference curve for each determination as fluctuations of temperature and length of incubation may change the slope of the reference curve.

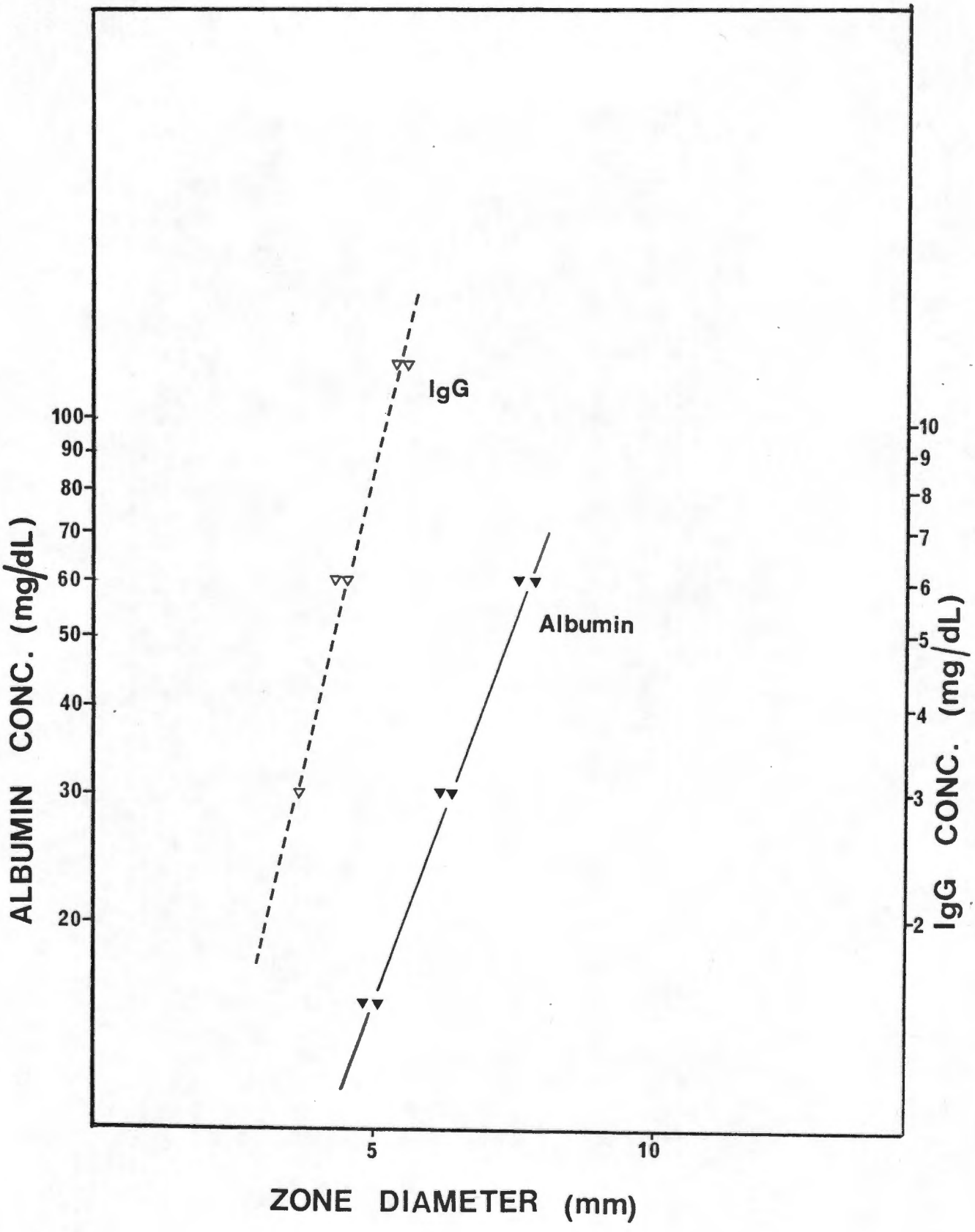
TABLE B.8 - ZONE DIAMETERS OBTAINED IN DUPLICATE FOR STANDARD SOLUTIONS OF IgG AND ALBUMIN BY RID

	CONCENTRATION (mg/dL)					
	IgG			ALBUMIN		
	3	6	12	3	6	12
ZONE DIAMETER (mm)	3,7	4,5	5,5	4,8	5,9	7,6
	3,7	4,4	5,4	5,1	8,2	7,9

FIGURE B.3



Standard curves of various concentrations of human albumin (▼——▼) and human IgG (▽- -▽) as determined by radial immunodiffusion.


IgG
Albumin
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
ALBUMIN CONC. (mg/dL)
IgG CONC. (mg/dL)
5
10
ZONE DIAMETER (mm)

B.5 DISCUSSION

Two different total protein determination methods, the Lowry method and the Coomassie Brilliant Blue method, were compared. They were both equally sensitive but the latter was a simpler method, requiring the addition of a single reagent and no incubation. Both methods showed colour variability with the proteins used. Unusual was the reverse in colour intensity of a single protein used in the Lowry and Coomassie Brilliant Blue methods. If pure IgG was used as a standard, there was an underestimation in CSF protein concentration by 25% with the Lowry method and an overestimation of 31% with the Coomassie Brilliant Blue method, when compared to the albumin/IgG mixture. The reason for this is unknown but may be due to the different reaction sites used by the various reagents.

ABBREVIATIONS

A	ß alanine
ABS	Absorbance
AC	Alternating current
ACTH	Adenocoricotropic Hormone
ADP	Adenosine diphosphate
Ala	β alanine
Alb.	Albumin
ALL	Acute lymphocytic leukaemia
Ammediol	2-amino-2-methyl-1,3 propandiol
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
c	Molar Concentration
C	Conductance
°C	Degree centigrade
Cap. tube	Capillary tube
CBB	Coomassie Brilliant Blue
Cl^-	Chloride ion
cm	Centimetre
cm^3	Cubic centimetre
Conc.	Concentration
Cps	Centipoise
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DATD	N'N diallytartardiamide

DC	Direct current
dL	Decilitre
DRX	Relative correction factor
E	Charge of an electron
E*	Field strength
$E^{1\%}_{1cm\,280nm}$	Extinction coefficient at 280 nm
ECHO	Enterocytopathic human orphan virus
EIP	Electrolyte impurity pattern
FRP	Front running peak(s)
G	Glycine
GBS	Guillain-Barré syndrome
H_3O+	Hydroxonium ion
HCl	Hydrochloric acid
Hf	High frequency
HPMC	Hydroxypropyl methylcellulose
hrs	Hours
I	Current
ID	Internal diameter
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgG/Alb	Immunoglobulin G to Albumin ratio
Intg.unit	Arbitary integrated units
K	Dissociation constant
Kcal	Calibration constant
kV	Kilovolts
L	Leucine

Leu	Leucine
log	Logarithm
m	Mobility
M	Molar
meff	Effective mobility
MES	2(N-morpholino) ethane sulphonic acid
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MS	Multiple Sclerosis
m/v	Mass to volume ratio
MW	Molecular weight
MZE	Multiphasic zone electrophoresis
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
nL	Nanolitre
nm	Nanometre
OH^-	Hydroxyl ion
pH	Log_{10} of the reciprocal of the hydrogen ion concentration
pI	Isoelectric point
pK	Log_{10} of the dissociation constant
pmole	Picomolar
R	Resistance
R*	Resolution

RID	Radial immunodiffusion
S	Velocity
SD	Standard deviation
SDS	Sodium dodecyl sulphate (Lauryl sulphate)
sec	Second
SSPE	Subacute sclerosing panencephalitis
Temp	Temperature
TES	N-tris-(hydroxymethyl)-methyl-2-aminoethane sulphonic acid
TP	Total protein
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet
V	Valine
V*	Velocity
val	Valine
v/v	Volume to volume ratio
X	Mean
x	Degree of dissociation
z	Elementary charge
EACA	Epsilon-n-amino caproic acid
µA	Microamperes
µL	Microlitre
µm	Micron
µM	Micromolar
%	Percentage
λ	Conductivity

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