# Retention model of protein for mixed-mode interaction mechanism in ion exchange and hydrophobic interaction chromatography

WEI, Yin-Mao*(卫引茂) LUO, Quan-Zhou(罗全舟)
LIU, Tong(刘彤) GENG, Xin-Du(耿信笃)
*Institute of Modern Separation Science, Northwest University, Xi'an, Shanxi 710069, China*

**A unified retention equation of proteins was proved to be valid for a mixed-mode interaction mechanism in ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC). The reason to form a "U" shape retention curve of proteins in both HIC and IEC was explained and the concentration range of the strongest elution ability for the mobile phase was determined with this equation. The parameters in this equation could be used to characterize the difference for either HIC or IEC adsorbents and the changes in the molecular conformation of proteins. With the parameters in this equation, the contributions of salt and water in the mobile phase to the protein retention in HIC and IEC were discussed, respectively. In addition, the comparison between the unified equation and Melander's three-parameter equation for mixed-mode interaction chromatography was also investigated and better results were obtained in former equation.**

**Keywords** Retention mechanisms, ion exchange chromatography, hydrophobic interaction chromatography, proteins

## Introduction

Ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC) have been widely employed in the separation of proteins. With the both, a three or four-dimensional structure and bioactivity of protein molecules generally maintain. The retention mechanisms of protein in the two kinds of chromatography have been presented in several models, such as electrostatic theory in IEC and solvophobic theory in HIC,[1] and stoichiometric displacement model[2] for the both, *etc*. During a process of protein separation, the retention curves often exhibit an "U" shape as salt concentration changes in mobile phase employed. This should be attributed to a mixed-mode interaction mechanism in both HIC and IEC. However, the general theory used in HIC and IEC often focuses on a single mechanism of retention. Therefore, they could not explain this phenomenon to form such a "U" shape curve reasonably. Melander *et al*.[1] proposed a three-parameter equation for elucidating this problem. According to the theory of stoichiometric displacement, one of the authors presented a unified retention equation for explaining this phenomenon of HIC in previous paper.[3] However, it did not discuss the physical meaning of each parameter in that equation and its application. In addition, this equation has not been tested with IEC. In this study, the unified equation was proved to be valid for IEC with the retention data in weak cation ion exchange chromatography (WCX), and the physical meaning of each parameter in the equation was proved by experiments. The comparison between the unified equation and Melander's three-parameter equation was also made.

## Theory

Considering all kinds of interactions in chromatographic system, such as the interactions between solute and solvent, solute and stationary phase, solvent and stationary phases and the competitive adsorption between salt and water molecules on the stationary phase employed, a retention equation was derived as:[3]

Received November 27, 1998; accepted August 12, 1999.
Project (Nos. 29675017 and 39880003) supported by the National Natural Science Foundation of China.

$$\log k' = \{\log K_5 + \log\phi + n(r + r')\log K_e\} - nr\log\{1 + K_e(m - q + 1)[W]\} - q\log[W] - (n^2r' + q')\log[S] \quad (1)$$

where, [W] and [S] denote the molar concentrations of water and salt in the empolyed mobile phase, respectively. $K_e$ and $m$ are the parameters relating to the chromatographic system used. The $nr$ and $nr'$ are the numbers of water and salt molecules released from the stationary phase at the interface between the stationary phase and the protein, respectively. The $q$ and $q'$ corresponding to $nr$ and $nr'$ represent the numbers of water and salt molecules released from the proteins at the interface between the stationary phase and the protein, respectively. The symbol $\phi$ is column phase ratio. The physical meanings of other parameters in Eq. (1) were explained in previous paper.[3] After some assumptions were done, Eq. (2) would be obtained as:[3]

$$\log k' = B_0 + B_1\log[W] + B_2[W] + B_3[W]^2 + B_4\log[S] \quad (2)$$

where,

$$B_0 = \log K_5 + \log\phi + n(r + r')\log K_e \quad (3)$$

$$B_1 = -q \quad (4)$$

$$B_2 = nr(m - q + 1)K_e \quad (5)$$

$$B_3 = 1/2nr(m - q + 1)^2K_e^2 \quad (6)$$

$$B_4 = -(n^2r' + q') \quad (7)$$

According to the physical meanings of $nr$, $q$, $q'$ and $r'$ pointed above, the three middle terms containing constants $B_1$, $B_2$, and $B_3$ on the right-hand side in Eq. (2) would be the contribution of hydrophobic interaction to the protein retention, while the last term, $B_4$ in it, be that of electrostatic interaction to protein retention.

## Experimental

*Equipment*

The chromatographic system (Shimadzu, Japan) consisting of two pumps (LC-6A), a system controller (SCl-6B), UV-Vis detector (SPD-6AV), and recorder wasemployed. Separations were carried out on HIC column (4.0 × 100 mm I.D), weak cation ion exchange (WCX) and weak anion ion exchange (WAX) column (7.9 × 50mmI.D), packed with XDF-1(7$\mu$m silica), WCX-1 and WAX-1 (7 $\mu$m polymer synthesized in our laboratory), respectively.

*Chemicals*

Cytochrome-C (Cyt-C), insulin (Ins), ribonuclease-A (Rnase-A), myoglobin (Myo), α-chymotrypsinogen A (α-Chy-A), and lysozyme (Lys) were purchased from Sigma Co. (St. Louis, Mo. USA).

*Chromatographic procedure*

Mobile phase was prepared with two pumps using following solutions: solution A, 3.0 mol/L ammonium sulphate and 20 mmol/L potassium dihydrogen phosphate (pH = 7.0); solution B, 20 mmol/L potassium dihydrogen phosphate (pH = 7.0). Before injecting a sample solution, the column must be equilibrated with 40 mL of the mobile phase. The flow rate was 1.0 mL/min and detection was at 280 nm.

The void volume of the chromatographic system was determined with $NaNO_2$ solution. Water concentration in mobile phase was calculated according to the following equation:

$$[W] = \frac{(d_A - d_B - M)S + 3.0d_B}{3.0 \times 0.018}(\text{mol/L}) \quad (8)$$

where, $d_A$ and $d_B$ are the densities of solutions A and B, respectively, $S$ is the concentration of ammonium sulphate in the mixed solution measured, $M$ is the amount of salt in solution A.

## Results and discussion

*Testing the unified equation*

The retention curves of Lys in WCX (Fig. 1a) and HIC (Fig. 1b) with isocratic elution mode were shown in Fig. 1, respectively. The retention factors of other five proteins at different salt concentrations in IEC and HIC were also listed in Table 1, respectively. The common features of Fig. 1, Tables 1 and 2 show that the protein retention gradually decreases with the increasing of salt concentration until to a minimum value denoted with underlines in two Tables and after that their retention increases. In other words, the retention curves of these proteins exhibit a "U" shape. This result is the same as that obtained by Melander *et al*.[1] and this phenomenon should be attributed to the multi-mode interaction mechanisms in HIC and IEC.

**Table 1** Retention factors for six proteins at different salt concentrations in HIC

| | Salt concentrations (mol/L) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Proteins | 0.025 | 0.038 | 0.050 | 0.075 | 0.125 | 0.25 | 0.50 | 0.75 | 1.00 | 1.25 | 1.50 | 1.75 | 2.00 |
| Myo | 0.22 | 0.30 | 0.01 | 0.07 | 0.05 | 0.04 | 0.04 | 0.04 | 0.07 | 0.14 | 0.31 | 0.62 | 3.22 |
| Rnase-A | 0.80 | 0.06 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.06 | 0.80 | 0.12 | 0.17 | 0.46 | 1.14 |
| Lys | 2.31 | 0.43 | 0.22 | 0.15 | 0.09 | 0.09 | 0.09 | 0.19 | 0.19 | 0.38 | 0.82 | 2.26 | 7.83 |
| Ins | – | – | – | 0.01 | 0.01 | 0.01 | 0.02 | 0.03 | 0.05 | 0.08 | 0.60 | 1.50 | 9.12 |
| α-Chy-A | – | – | – | 0.01 | 0.01 | 0.01 | 0.02 | 0.05 | 0.06 | 0.13 | 0.38 | 1.32 | 7.33 |
| Cyt-C | 0.54 | 0.18 | 0.17 | 0.08 | 0.05 | 0.04 | 0.04 | 0.05 | 0.05 | 0.08 | 0.12 | 0.15 | 0.18 |

**Table 2** Retention factors for six proteins at different salt concentrations in IEC

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $C$ (mol/L) | | 0.06 | 0.07 | 0.08 | 0.9 | 0.12 | 0.15 | 0.18 | 1.8 | 1.83 | 1.92 | 1.98 | 2.04 |
| Myo | $k'_e$ | 8.32 | 3.99 | 2.72 | 1.82 | 0.89 | 0.57 | 0.41 | 0.45 | 0.83 | 0.90 | 1.57 | 1.83 |
| | $k'_c$ | 6.76 | 4.32 | 2.96 | 2.10 | 0.97 | 0.54 | 0.34 | 0.63 | 0.71 | 1.02 | 1.30 | 1.68 |
| | $d$ | 1.56 | -0.33 | -0.24 | -0.28 | -0.08 | 0.03 | 0.07 | -0.18 | 0.12 | -0.12 | 0.27 | 0.23 |
| Rnase-A | $k'_e$ | 2.96 | 1.73 | 1.2 | 0.70 | – | – | – | 0.92 | 1.07 | 1.71 | 2.36P | 3.19 |
| | $k'_c$ | 2.18 | 1.60 | 1.22 | 0.95 | – | – | – | 0.67 | 0.83 | 1.68 | 2.75 | 4.62 |
| | $d$ | 0.78 | 0.13 | -0.02 | -0.25 | – | – | – | 0.25 | 0.24 | 0.03 | -0.39 | 1.43 |
| $C$ (mol/L) | | 0.21 | 0.27 | 0.3 | 0.36 | 0.45 | 0.51 | 0.6 | 0.81 | 0.99 | 1.14 | 1.2 | 1.32 |
| Lys | $k'_e$ | 2.10 | 1.15 | 0.49 | 0.75 | 0.51 | 0.55 | 0.46 | 0.62 | 0.88 | 1.50 | 1.60 | 2.5 |
| | $k'_c$ | 2.06 | 1.18 | 0.86 | 0.71 | 0.55 | 0.50 | 0.49 | 0.60 | 0.90 | 1.39 | 1.68 | 2.54 |
| | $d$ | 0.04 | -0.03 | -0.35 | 0.04 | -0.04 | 0.05 | -0.03 | 0.02 | -0.02 | 0.11 | -0.08 | -0.04 |
| Ins* | $k'_e$ | 0.63 | 0.60 | 0.49 | 0.30 | 0.28 | 0.28 | 0.26 | 0.35 | 1.23 | 1.93 | 2.89 | 7.00 |
| | $k'_c$ | 0.87 | 0.49 | 0.40P | 0.31 | 0.26 | 0.26 | 0.28 | 0.50 | 1.05 | 2.19 | 3.00 | 5.85 |
| | $d$ | -0.24 | 0.11 | 0.09 | -0.01 | 0.02 | 0.02 | -0.02 | -0 | 15 | 0.18 | -0.06 | -0.11 | 1.15 |
| $C$ (mol/L) | | 0.09 | 0.18 | 0.24 | 0.3 | 0.42 | 0.48 | 0.54 | 0.6 | 0.66 | 0.72 | 0.81 | 0.99 |
| α-Chy-A | $k'_e$ | 15.8 | 1.39 | 0.75 | 0.47 | 0.35 | 0.40 | 0.50 | 0.56 | 0.71 | 0.93 | 1.58 | 4.05 |
| | $k'_c$ | 14.7 | 1.42 | 0.70 | 0.48 | 0.38 | 0.40 | 0.46 | 0.55 | 0.69 | 0.91 | 1.43 | 4.13 |
| | $d$ | 1.1 | -0.03 | 0.05 | -0.01 | -0.03 | 0.00 | 0.04 | 0.01 | 0.02 | 0.02 | 0.15 | -0.08 |
| $C$ (mol/L) | | 0.09 | 0.12 | 0.15 | 0.21 | 2.34 | 2.4 | 2.46 | 2.49 | 2.52 | 2.58 | 2.64 | 2.73 |
| Cyt-C | $k'_e$ | 5.68 | 1.94 | 0.78 | 0.24 | 0.59 | 0.74 | 0.92 | 1.03 | 1.15 | 1.46 | 1.82 | 2.61 |
| | $k'_c$ | 5.83 | 1.86 | 0.80 | 0.24 | 0.60 | 0.74 | 0.93 | 1.03 | 1.16 | 1.45 | 1.82 | 2.56 |
| | $d$ | -0.15 | 0.08 | -0.02 | 0.00 | -0.01 | 0.00 | -0.01 | 0.00 | -0.01 | 0.01 | 0.00 | 0.05 |

The $k'_e$, and $k'_c$ denote the experimental and calculated value, respectively. * The stationary phase is WAX-1.

Table 3 lists the parameters calculated by Eq. (2). The standard deviations of the experimental data of

the six proteins in WCX and HIC were obtained from the least square analysis, respectively. In order to compare the experimental value and the calculated value, Table 2 also shows the calculated retention factors and deviation in IEC with Eq. (2). Most of the deviations in Table 2 are less than 10% and all of the standard deviations in Table 3 are less than 0.4. These results elucidate the experimental data fits Eq. (2) well. The elution curves of Lys calculated by Eq. (2) shown by the triangle symbol in Fig. 1 also indicate the foregoing points. Thus, it could be concluded that Eq. (2) effectively coincides the multi-mode interaction mechanisms in both HIC and IEC.

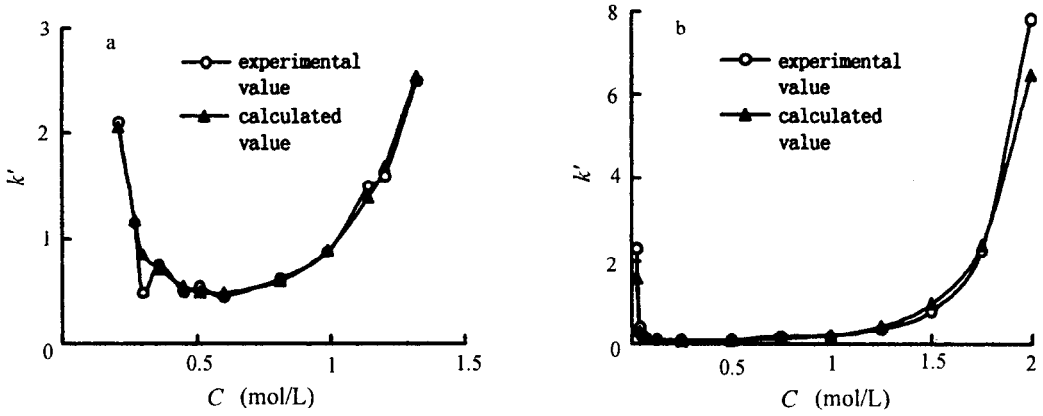


**Fig. 1** Retention curves of Lys in IEC (a) and HIC (b).

**Table 3** Parameters (Eq. 2) and deviations for six proteins in HIC and IEC

| | | $B_0$ | $B_1$ | $B_2$ | $B_3$ | $B_4$ | Std |
|---|---|---|---|---|---|---|---|
| Myo | HIC | 118.36 | -56.60 | -1.15 | 0.02 | -0.94 | 0.11 |
| | IEC | 61.99 | 6.67 | -2.22 | 0.02 | -3.16 | 0.07 |
| Rnase-A | HIC | 169.95 | -200.77 | 5.67 | -0.05 | -1.28 | 0.35 |
| | IEC | 87.94 | 185.86 | -13.78 | 0.11 | -1.87 | 0.11 |
| Lys | HIC | 77.12 | -38.96 | -0.69 | 0.01 | -1.03 | 0.09 |
| | IEC | 17.47 | 13.71 | -0.71 | -0.00 | -3.66 | 0.02 |
| Ins | HIC | 238.88 | -119.37 | -3.10 | -0.05 | 0.52 | 0.10 |
| | IEC* | 3.96 | 46.72 | -1.77 | 0.03 | -4.45 | 0.08 |
| α-Chy-A | HIC | 106.01 | -12.70 | -3.92 | 0.04 | 0.40 | 0.09 |
| | IEC | 22.70 | -2.44 | 0.62 | -0.02 | 4.97 | 0.03 |
| Cyt-C | HIC | 103.81 | -117.84 | 3.16 | -0.03 | -1.32 | 0.08 |
| | IEC | 30.26 | 0.09 | -0.57 | -0.00 | -4.56 | 0.01 |

Std-standard deviation. * The meaning is the same as in Table 2.

*Comparative contribution of water and salt to protein retention*

It should be firstly explained the reason why the retention curve of proteins in both IEC and HIC has a "U" shape. According to the foregoing calculated result, the contributions of both water for hydrophobic mechanism and salt for ion exchange mechanism to the protein retention would be conformed, respectively.

From Eqs. (5) and (6), the relationship between $B_2$ and $B_3$ is as:

$$nr = B_2^2/2B_3 \quad (9)$$

From Table 3, it can be seen that $|B_2|$ (absolute value) is much larger than $|B_3|$. Therefore, the value

of $nr$ should be large enough. The parameter, $q$ [$B_1$ in Eq. (2)], as shown in Table 3, is also a large value. As a result, the second and third parameters pointed out above in Eq. (1) are greater than the last term in the right-hand side of this equation. The second and the third terms in Eq. (1) are parameters relating to the hydrophobic interactions, and the last term, $(n^2r' + q')$ [equals to $B_4$ in Eq. (2)] to electrostatic interaction. Since the [W] in Eq. (1) is much greater than [S] in mobile phase, it may be expected that water is a dominant factor contributing to protein retention even though salt concentration in it very high in both HIC and IEC. In other words, the hydrophobic interactions play a main role to protein retention in higher concentration of salt. On the contrary, when salt concentration is very low (less than tens mmol/L for HIC and 1.0 mol/L for IEC), log[S] in Eq. (1) would become a large value. As a result, the salt contribution to the protein retention would increase. So the elution curve of proteins was observed to appear a "U" shape.

It should be next elucidated that the contributions of electrostatic force in IEC and hydrophobic force in HIC would dominate protein retentions, respectively. In other words, though they have the same mixed retention model and could be expressed with the same Eq. (1) or Eq. (2), the difference between the two kinds of stationary phases still exits. The data in Table 3 also show the values of $|B_4|$ for HIC being much less than those for IEC. This exactly confirms to the fact that the electronic interaction between protein and the adsorbent in HIC is less than that in IEC, because of the less electronic density on HIC adsorbent surface. It also consists with the physical meaning of the two terms in Eq. (2). Thus, $B_4$ would be used as a parameter to characterize the difference between HIC and IEC adsorbent. On the other hand, from Eqs. (1) and (2), the value of $(nr + q)$ is equal to $B_2^2/2B_3 - B_1$ and represents the numbers of water molecules released from the interface between the stationary phase and the protein.[3] Both $(nr + q)$ and the parameter, $B_4$[or $(n^2r' + q')$ in Eq. (1)] in Eq. (2) are related to the interface region between the stationary phase and solute molecules. Just as $Z$ in SDM-R,[4] both $(nr + q)$ and $B_4$[or $(n^2r' + q')$ in Eq. (1)] in Eq. (2) could also be referred to a characterization parameter for the changes in the molecular conformation of proteins.

The third question should be answered that why the salt concentrations of the mobile phase were selected to be different for IEC and HIC by using the unified equation. From Eq. (2), the concentration of water or salt at the minimum of $k'$ in HIC and IEC, $[W]_{min}$ or $[S]_{min}$, could be obtained. $[W]_{min}$ and $[S]_{min}$ denote the water and salt concentrations to have the strongest elution ability of the mobile phase employed, respectively. The $[S]_{min}$ values in HIC and IEC were listed in Table 4. For the six proteins, the range of $[S]_{min}$ in HIC and IEC are 0—0.45 and 0—0.88 mol/L, respectively. When [S] is more than $[S]_{min}$, the contribution of hydrophobic interaction to the protein retention was found to be stronger than that of the electronic interaction. In the circumstance of $[S] < [S]_{min}$, it is just the opposite. That is the reason why the salt concentration in mobile phase should often be more than 0.5 mol/L in the HIC, while less than 1.0 mol/L in IEC.

**Table 4** $[S]_{min}$ values in HIC and IEC (mol/L)

| | Myo | Ins | Rnase-A | Lys | Cyt-C | α-Chy-A |
|---|---|---|---|---|---|---|
| HIC | 0.35 | 0.24 | 0.09 | 0.45 | 0.45 | 0.31 |
| IEC | 0.64 | 0.49* | 0.56 | 0.59 | 0.88 | 0.42 |

* The meaning is the same as in Table 2.

*Comparison between the unified Eq. (2) and Melander's equation*

Three-parameter equation for mixed-mode interaction mechanism of protein in IEC proposed by Melander *et al*.[1] is shown as follows:

$$\log k' = A - B\log m + Cm \qquad (10)$$

where $m$ is the salt concentration in mobile phase, $B$ and $C$ are the appropriate electrostatic and hydrophobic interaction parameters, respectively, and $A$ is a constant. The calculated data based on Eq. (10) were listed in Table 5.

Compared to the data in Table 3 and Table 5, it shows that the Melander's equation has larger deviation than that of the unified equation of the SDM-R. Therefore, it would be concluded that the later equation is better than the former.

In the experiment, as that pointed above, the ionic interaction causing from HIC column is weaker than that from the IEC column, but the hydrophobicity from the former is stronger than that from the latter. The values of

$B$ in Eq. (10) in IEC are higher than those in HIC. This result reflects their natures of the HIC and IEC column themselves, respectively. However, the values of $C$ in IEC are also higher than those in HIC, this result could not reflect the experimental data, and is not consistent with the physical meaning in parameter $C$. This may be attributed to the fact that Eq. (10) is based on the solvephobic theory based on surface tension to be an oversimplication when dealing with such a complex system protein binding.

**Table 5** Retention parameters (Eq. 10) for HIC and IEC

| | Myo | | Rnase-A | | Lys | | Cyt-C | | α-Chy-A | | Ins | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HIC | IEC | HIC | IEC | HIC | IEC | HIC | IEC | HIC | IEC | HIC | IEC* |
| A | -2.80 | -3.34 | -2.26 | -4.75 | -2.35 | -2.82 | -2.20 | -4.20 | -1.54 | -4.30 | -3.42 | -3.89 |
| B | 1.40 | 3.32 | 0.89 | 4.12 | 1.29 | 3.76 | 1.12 | 4.54 | 0.14 | 4.79 | 1.22 | 4.43 |
| C | 1.74 | 2.24 | 1.20 | 3.2 | 1.74 | 2.78 | 1.00 | 2.42 | 0.68 | 4.95 | 2.25 | 3.94 |
| Std | 0.13 | 0.08 | 0.37 | 0.20 | 0.10 | 0.08 | 0.10 | 0.01 | 0.35 | 0.04 | 0.18 | 0.08 |

* The meaning is the same as in Table 2.

## Conclusion

1. A unified retention equation of proteins from stoichiometric displacement model was proved to be valid for a mixed-mode interaction mechanism in ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC). With the parameters in this equation, it may be expected that water is a dominanted factor for protein retention, while, at low salt concentration, electrostatic force mainly contributes to the protein retention. As a result, the elution curves of proteins in IEC and HIC were observed to exhibit a "U" shape.
2. The term $(nr + q)$ and the parameter, $B_4$ in this equation could be used to characterize the difference between either HIC or IEC adsorbents and the changes in the molecular conformation of proteins.
3. The concentration range of the strongest eluted ability for the mobile phases in both IEC and HIC can be determined with the unified equation.
4. It was found that the unified equation presented this paper fits the experiment facts better than by Melander's three-parameter equation.

## References

1. Melander, W.R.; Corradini, D.; Horvath, C., *J. Chromatogr.*, **317**, 67(1984).
2. Geng, X.D; Guo, L.A; Chang, J.H., *J. Chromatogr.*, **507**, 1(1990).
3. Bian, L.J; Feng, W.K; Geng, X.D., *Chin. J. Chromatogr.* (in Chinese), **9**, 267(1991).
4. Shi, Y.L.; Ma, F.; Geng X.D., *Fenxi Huaxue* (in Chinese), **22**, 453(1994).

(SONG, J.P.; DONG, L.J)